MOLECULAR STUDIES OF *KAPPAPHYCUS* DOTY AND *EUCHEUMA* J. AGARDH: PHYLOGENETICS AND DNA BARCODE ASSESSMENT

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ABSTRACT

Kappaphycus and *Eucheuma* are the main producers of carrageenan worldwide, with stable annual production increments to cater to increasing demands. Extensively used in the food and cosmetics industries, the marketing of carrageenan generates lucrative returns to the industry and economy. The carrageenan industry is one of the key economic sectors in Malaysia, which also offers a means of livelihood to the local community. The extensive morphological variations of *Kappaphycus* and *Eucheuma* often resulted in faming of mixed populations which reduced overall carrageenan yields. Molecular taxonomy is thus applied to identify the many locally-named varieties of *Kappaphycus* and *Eucheuma* as well as elucidate the phylogeny associated with these red seaweeds. Local varieties, categorized via putative external morphology, were analyzed using the mitochondrial *cox*2-3 spacer and RuBisCO spacer DNA markers. The *cox*2-3 spacer provided better phylogenetic delineation compared to the RuBisCO spacer. Results revealed that morphological and color variations are unsupported by genetic data, where many of the local varieties of *Kappaphycus* and *Eucheuma* are invalid. Phylogenetics has also shown the genetic distinctiveness of two *K. alvarezii* genotypes exclusive to Hawaii and Africa that differs from the commonly cultivated *K. alvarezii* available worldwide. Two genetically different strains of *K. striatus* were also observed in Malaysia. The local variety *Kappaphycus* "*Aring-aring*" displayed unique phenotypic and genotypic traits and may possibly be a new species. *E. denticulatum* was shown to be dominant in East Malaysian waters, where the "*Spinosum*" and "*Cacing*" varieties differ from one another both in terms of morphology and genetics. The "*Cacing*" variety was shown to be synonymous with *E. denticulatum* (Burman) Collins & Hervey var. *endong* Trono & Ganzon-Fortes var. nov. The paraphyletic nature of *Eucheuma* was also shown and discussed.

The usefulness of molecular taxonomy encouraged the assessment of potential molecular markers for DNA barcoding of the rhodophytes *Kappaphycus* and *Eucheuma* on a larger scale. Proper establishments of DNA barcode libraries of these commercially important seaweeds would hasten species identifications, phylogenetic inferences, biodiversity studies, population studies, bioinvasion monitoring as well as the identification and selection of superior strains for cultivation. The effectiveness in DNA barcoding of four genetic markers, namely the mitochondrial *cox*1, *cox*2, *cox*2-3 spacer and the plastid *rbc*L were gauged using a dataset comprised of selected *Kappaphycus* and *Eucheuma* samples from Southeast Asia. Marker assessments were performed using established distance and tree-based identification criteria from earlier studies. Barcoding patterns on a larger scale were simulated by empirically testing on the commonly used *cox*2-3 spacer. The *cox*2 marker which satisfies the prerequisites of DNA barcodes was found to exhibit moderately high interspecific divergences with no intraspecific variations, thus a promising marker for the DNA barcoding of *Kappaphycus* and *Eucheuma*. However, the already extensively used *cox*2-3 spacer was deemed to be in overall more appropriate as a DNA barcode for these two genera. On a wider scale, *cox*1 and *rbc*L were still better DNA barcodes across the rhodophyte taxa when practicality and cost-efficiency were taken into account. The application of DNA barcoding has demonstrated our relatively poor taxonomic comprehension of these seaweeds, thus suggesting more in-depth efforts in taxonomic restructuring.

ABSTRAK

Rumpai laut *Kappaphycus* dan *Eucheuma* merupakan sumber utama karaginan di seluruh dunia, dengan peningkatan tahunan yang stabil bagi memenuhi keperluan khususnya daripada industri makanan dan kosmetik. Oleh hal yang demikian, industri karaginan telah ditentukan sebagai salah satu komoditi ekonomi penting di Malaysia yang banyak menawarkan peluang pekerjaan terutamanya kepada komuniti miskin. Namun demikian, sifat plastik dari segi morfologi *Kappaphycus* dan *Eucheuma* yang mengelirukan sering mengakibatkan penanaman rumpai laut secara tercampur oleh para petani. Hal ini menjurus kepada penurunan pengeluaran karaginan yang serius. Taksonomi molekular telah diperkenalkan untuk membezakan varieti-varieti tempatan *Kappaphycus* dan *Euchema* serta mentafsirkan hubungan filogenetik antara alga merah tersebut. Varieti-varieti tempatan telah dikumpulkan dan dikategorikan berdasarkan ciri-ciri morfologi luaran yang dibekalkan oleh para-petani dan kemudian ditaklukan kepada analisis DNA dengan menggunakan penanda molekular *cox*2-3 spacer mitokondria dan RuBisCO spacer plastida. Keputusan analisis menunjukkan bahawan resolusi filogenetik *cox*2-3 spacer lebih spesifik daripada RuBisCO spacer, dan variasi-variasi warna serta morfologi yang diperhatikan pada kebanyakan varieti *Kappaphycus* dan *Eucheuma* tidak disokong oleh data molekular. Keputusan filogenetik turut memaparkan genotip unik *K. alvarezii* dari Afrika dan Hawaii yang berlainan daripada genotip kultivar *K. alvarezii* yang biasanya ditanam keseluruhan dunia. Dua genotip berbeza bagi *K. striatus* juga dikesan berdasarkan DNA. Varieti tempatan "*Aring-aring*" yang menunjukkan ciri-ciri fenotip dan genotip yang unik berpotensi sebagai spesis baru *Kappaphycus*. Analisis molekular turut menunjukkan bahawa *E. denticulatum* merupakan spesis dominan dalam laut Malaysia Timur, di mana varieti "*Spinosum*" dan "*Cacing*" berbeza daripada satu sama lain dari segi morfologi dan juga genetik. Identiti varieti "*Cacing*" telah dikenalpastikan sebagai *E. denticulatum* (Burman) Collins &

Hervey var. *endong* Trono & Ganzon-Fortes. Sifat parafiletik genus *Eucheuma* juga dibincangkan.

Kepentingan taksonomi molekular telah mendorong pengujian penanda-penanda molekular yang berpotensi sebagai barkod DNA bagi rumpai laut *Kappaphycus* dan *Eucheuma* secara besar-besaran. Penubuhan *DNA barcode library* yang lengkap memainkan peranan yang penting dalam kepantasan identifikasi spesis, penganggaran inferens filogentik, kajian biodiversiti dan populasi, pemantauan bio-pencerobohan serta identifikasi dan pemilihan baka yang baik untuk kultivasi. Keberkesanan *DNA Barcoding* bagi penanda-penanda molekular *cox*1, *cox*2, *cox*2-3 spacer dan *rbc*L telah diuji dengan menggunakan sampel-sampel *Kappaphycus* dan *Eucheuma* terpilih dari Asia Tenggara. Penanda-penanda molekular tersebut ditaklukan kepada kriteria identifikasi *tree-based* dan *distance-based* yang dicadangkan oleh kajian terdahulu. Kejituan *DNA Barcoding* bagi skala yang lebih besar turut disimulasikan secara empirical dengan menggunakan *cox*2-3 spacer mitokondria. Keputusan telah merumuskan bahawa penanda molekular *cox*2 berpotensi sebagai barkod DNA bagi *Kappaphycus* dan *Eucheuma* kerana memenuhi prasyarat-prasyarat yang ditentukan, dan pada masa yang sama menunjukkan perbezaan interspesifik yang agak tinggi serta ketiadaan perbezaan intraspesifik. Namun begitu, penanda molekular *cox*2-3 spacer yang lebih universal dan popular dianggap lebih sesuai bagi *DNA Barcoding Kappaphycus* dan *Eucheuma*. Dari segi *DNA Barcoding* bagi taxa yang lebih luas, *cox*1 dan *rbc*L merupakan penanda-penanda molekular yang lebih praktikal dan kos efektif secara keseluruhan. Aplikasi *DNA Barcoding* juga menunjukkan kelemahan ilmu taksonomi yang sedia ada bagi *Kappaphycus* dan *Eucheuma*. Hal ini menyeru kajian lebih mendalam bagi tujuan penambahbaikan dan penstrukturan taksonmi.

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Adenine
ASB	*All Species Barcode*
BM	*Best Match*
BCM	*Best Close Match*
BOLD	Barcode of Life Data System
bp	Base Pair
C	Cystosine
CI	Consistency Index
cm	centimeter
cox	Cytochrome *c* Oxidase
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
FAO	Food and Agriculture Organization
G	Guanine
ITS	Internal Transcribed Spacer
K2P	Kimura-2-Parameter
Kb	Kilobase
kg	Kilograms
LSU	Large Subunit
m	meter
ML	Maximum Likelihood
MP	Maximum Parsimony
µl	Microliter

µg	Microgram
MSA	Multiple Sequence Alignment
ng	Nanogram
NJ	Neighbor-Joining
PCR	Polymerase Chain Reaction
pmol	Picomole
PES	Processed *Eucheuma* Seaweed
PNG	Philippine Natural Grade
PVC	Polyvinyl Chloride
*rbc*L	Large Subunit of RuBisCO
rDNA	Ribosomal Deoxyribonucleic Acid
RI	Retention Index
rpm	Revolutions per Minute
rRNA	Ribosomal Ribonucleic Acid
RuBisCO	Ribulose-1,5-Bisphosphate Carboxylase Oxygenase
SRC	Semi-Refined Carrageenan
SSU	Small Subunit
T	Thymine
TAE	Tris-Acetate-EDTA
T_m	Melting Temperature
TAE	Tris-Acetate-EDTA
Temp.	Temperature
UPA	Universal Plastid Amplicon
US	United States
UV	Ultraviolet
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 An overview of the commercially important *Kappaphycus* and *Eucheuma*

Rhodophytes are economically important seaweeds highly valued for the hydrocolloids they produce, generating substantial amounts of revenue in the global market. Fetching up to 700 million US dollars as of year 2009 (an approximate 67% increment over a decade), the global seaweed hydrocolloid sales value continued to record stable growth, with the mushrooming of seaweed farms throughout tropical areas of the world (Bixler 1996; Bixler and Porse 2010). Carrageenan, a sulfated polysaccharide exhibiting gel-forming and viscosifying properties, remains the most widely demanded hydrocolloid as of today. Owing to its gelling and thickening properties, carrageenan is extensively used in food, pharmaceutical and cosmetic industries (McHugh 2003a; Pereira et al. 2007) and depending on the processing method, one kilogram of carrageenan could cost six to fifteen US dollars in 2009 (Bixler and Porse 2010).

Carrageenan is found in cell walls of red seaweeds within the family Gigartinales (Pereira et al. 2007; Pereira and Velde 2011) which is believed to have first been discovered by accident during the 16th century (West 2001), and the knowledge has since then been introduced throughout the globe. *Chondrus crispus* Stackhouse (Irish Moss) was the first sole source of carrageenan before 1975 due to the available wild stocks (Lobban and Harrison 1996; West 2001). As wild populations begin to dwindle, cultivation efforts ensued around the 1970s in order to meet the increasing demands for the hydrocolloid; this was when the more robust and rapid-growing *Kappaphycus* Doty and *Eucheuma* J. Agardh of the family Solieriaceae were introduced (Doty 1985; Doty and Norris 1985). Anecdotally believed to originate from the Philippines, these seaweeds thrived and were very popular, eventually introduced and henceforth vegetatively propagated into other tropical parts of the world e.g. Africa,

China, Columbia, Fiji, Hawaii, India, Indonesia, Malaysia, Mexico, Singapore, Vietnam etc. for commercial cultivation (Ask and Azanza 2002; Ask et al. 2003; Bindu and Levine 2010; Bixler and Porse 2010; Hayashi et al. 2007; Munoz et al. 2004; Neish 2003; Paula et al. 1999; Phang et al. 2010; Pickering 2006). *Kappaphycus alvarezii*, *Kappaphycus striatus* and *Eucheuma denticulatum* have since then been extensively farmed. Despite the broad distribution of cultivation sites worldwide, Indonesia and the Philippines are to date the largest producers of carrageenan, accounting for more than 90% of the global carrageenan production (Bixler and Porse 2010).

The lucrative businesses associated with the cultivation of *Kappaphycus* and *Eucheuma* have led to many studies, including studies on growth parameters (Gerung and Ohno 1997; Góes and Reis 2011; Hurtado et al. 1996; Hurtado et al. 2001; Hurtado et al. 2008; Munoz et al. 2004; Thirumaran and Anantharaman 2009), epiphytes (Borlongan et al. 2011; Hurtado et al. 2006; Neish 2003; Vairappan 2006), tissue culture (Dawes and Koch 1991; Hurtado and Biter 2007), carpospore culture (Ask et al. 2001; Luhan and Sollesta 2010), tetraspore culture (Bulboa et al. 2008; Bulboa et al. 2007; Paula et al. 1999) and even development of hybrids (Cheney et al. 1998). Apart from growth optimization and strain improvement, *Kappaphycus* and *Eucheuma* seaweeds were also desired for their lectin content (Hung et al. 2008), enhanced immunostimulatory and antitumor activity (Yuan and Song 2005; Yuan et al. 2010), and also potential bioethanol production (Meinita et al. 2011). However, despite these advancements, fundamental taxonomic studies on these two red seaweeds have been limited, mostly because of their morphologically plastic nature (Bindu and Levine 2010; Conklin et al. 2009; Neish 2003; Zuccarello et al. 2006; Doty 1985; Doty and Norris 1985; Ganzon-Fortes et al. 2011). Distinguished solely based on external morphology by native farmers, large numbers of local names arose, eventually leading to confusion in identification and cultivation of these carrageenophytes (Neish 2003; Zuccarello et al.

2006; Ganzon-Fortes et al. 2011). Farming of mixed populations of *Kappaphycus* and *Eucheuma* will inevitably decrease optimal yield as the former produces *kappa* (κ) carrageenan, whereas the latter produces *iota* (ι) carrageenan (Doty and Norris 1985; Ganzon-Fortes et al. 2011): carrageenan-processing factories require clear separation of these two carrageenophytes prior to carrageenan extraction due to their varying gelling properties. The additional workforce employed to sort out *Kappaphycus* and *Eucheuma* seaweeds would incur higher costs to the industry.

Seeing the knowledge gap pertaining to the taxonomy of *Kappaphycus* and *Eucheuma* as well as the potential productivity loss due to misplantations, scientists have applied molecular approaches in hopes to better understand the identity and phylogenetic relations as well as the distribution of these red algae throughout the globe (Conklin et al. 2009; Dang et al. 2008; Ganzon-Fortes et al. 2011; Halling et al. 2012; Montes et al. 2008; Zuccarello et al. 2006). Genetic markers, specifically the mitochondrial *cox*2-3 spacer and the plastid-encoded RuBisCO spacer, were shown by Zuccarello and co-workers (2006) to be capable of delineating species of *Kappaphycus* and *Eucheuma* to a certain extent. This study has since paved way for subsequent molecular taxonomy work using other molecular markers, all of which returned promising results (Conklin et al. 2009; Ganzon-Fortes et al. 2011; Halling et al. 2012; Zhao and He 2011). While these scientists within the field continued to progress, further unraveling the phylogeny of *Kappaphycus* and *Eucheuma*, other groups of molecular systematists were developing DNA barcodes. First introduced by Herbert and co-workers (2003a; 2003b; 2004), DNA barcoding employs the usage of short, easily amplified DNA region(s) that exhibit large variation among species, yet are sufficiently variable within species, for species delineation and identification, as well as archiving with reference to known, established species (Ellegren et al. 2008; Hollingsworth et al. 2011; Jinbo et al. 2011). The Barcode of Life Data System (BOLD) is notably the

largest initiative in establishing a worldwide DNA barcode library, signifying its importance and popularity for the scientific community (Ratnasingham and Hebert 2007; Sarkar and Trizna 2011; Wong et al. 2011).

Although DNA barcoding was initially used for animals, the promising benefits eventually led to its application to other organisms, including algae. The usefulness of DNA barcoding is especially apparent when dealing with taxa displaying phenotypic plasticity throughout diphasic or triphasic life cycles as well as taxa involving cryptic species, often observed in marine macroalgae: The application of DNA barcoding has been reported in numerous studies encompassing the orders Gelidiales (Freshwater et al. 2010), Gigartinales (Clarkston and Saunders 2010; Le Gall and Saunders 2010; Saunders 2008), Graciliariales (Kim et al. 2010; Saunders 2009), Laminariales (McDevit and Saunders 2010), and Fucales (Kucera and Saunders 2008). DNA barcoding on wider taxa of rhodophytes have also been conducted with auspicious results (Robba et al. 2006; Saunders 2005). However, *Kappaphycus* and *Eucheuma* were scarcely covered in most of the previous DNA barcoding researches, thereby encouraging the development and assessment of suitable DNA barcodes for these carrageenophyes. Apart from enabling phylogenetic inference, species identification and biodiversity studies (Jinbo et al. 2011); application of DNA barcoding will also facilitate selection of superior strains as well as the monitoring of growth patterns and distribution of commercially introduced, potentially invasive *Kappaphycus* and *Eucheuma*, so as to avoid uncontrolled dispersion which might affect the native biota (Conklin et al. 2009; Halling et al. 2012; Zuccarello et al. 2006).

In spite of these technological advancements and the promising prospects, the genetic mapping and archiving of *Kappaphycus* and *Eucheuma* in the Southeast Asia remained limited prior to the research reported here. This is an impediment to the better understanding of the overall biodiversity, genetic diversity and phylogeny of these red

seaweeds, which is important because (1) Southeast Asia, particularly the Coral Triangle, is known to be a marine biological hotspot, with many organisms (be it plant or animal) yet to be identified (Veron et al. 2011); (2) Indonesia and the Philippines are the largest *Kappaphycus* and *Eucheuma* producers, with ample amounts of potentially different strains; and (3) the very first commercial tropical carrageenophytes were originated from the Philippines, which may help in tracing back the ancestry of these red algae. These reasons indicate an urgent need to employ molecular systematics on *Kappaphycus* and *Eucheuma* from Southeast Asia.

In Malaysia, the farming of *Kappaphycus* and *Eucheuma*, which was believed to have been introduced from the Philippines, started approximately four decades ago (Phang et al. 2010; Vairappan 2006). Concentrated along the Sabah coastline (Semporna, Kudat, Kunak, Banggi, Lahad Datu) (Phang et al. 2010), the cultivation of these seaweeds has long provided a source of income for the local communities, particularly the poor. Increasing demands for carrageenan have led to their introduction, albeit on a smaller scale, to the Pangkor and Langkawi islands of Peninsular Malaysia. Malaysia produced 15,000 tons of dried carrageenan in 2010, a 2,000 ton increase since 2009 (personal communication from Adibi Rahiman B. Md. Nor, officer from Department of Fisheries Malaysia). Despite the notable increase in *Kappaphycus* and *Eucheuna* farms in Malaysia, scientific studies on these carrageenophytes were slow and limited. Additionally, as a result of morphological plasticity, a large number of varieties were established and used by local farmers, not knowing whether these varieties were of the same species. These varieties were allegedly named on the basis of external morphology and color, including *Tambalang Brown*, *Tambalang Green*, *Tambalang Pink*, *Tambalang Giant*, *Tambalang Buaya*, *Tangan-tangan* (Loving Beauty), *Green Flower*, *Yellow Flower*, *Aring-aring*, *Cacing* and *Spinosum* (Phang et al. 2010). Local names are believed to be unreliable and inaccurate for distinguishing

varieties or even species, often leading to the issue of planting mixed populations by farmers as aforementioned. This study, which applies molecular taxonomy and DNA barcoding, was designed to resolve the said issues.

1.2 Objectives of research

The hypotheses to be examined are:

(a) Molecular taxonomy of *Kappaphycus* and *Eucheuma* in Malaysia

Hypothesis 1:

$\boldsymbol{H_0}$**:** The Malaysian varieties of *Kappaphycus* are conspecific.

$\boldsymbol{H_A}$**:** The Malaysian varieties of *Kappaphycus* are not conspecific.

Hypothesis 2:

$\boldsymbol{H_0}$**:** The Malaysian varieties of *Eucheuma are* conspecific.

$\boldsymbol{H_A}$**:** The Malaysian varieties of *Eucheuma* are not conspecific.

(b) Molecular marker assessments for DNA barcoding of *Kappaphycus* and *Eucheuma*

Research Question:

Is DNA barcoding applicable to the genera *Kappaphycus* and *Eucheuma*?

The objectives of this study are:

1. To elucidate the taxonomic confusion associated with the varieties of *Kappaphycus* and *Eucheuma* in Malaysia
2. To determine the phylogenetic relationship between Malaysian varieties of *Kappaphycus* and *Eucheuma* and those from within and outside Malaysia
3. To develop and assess potential DNA barcode(s) for *Kappaphycus* and *Eucheuma*.

A flow chart showing the research approach for this study is provided in Figure 1.1.

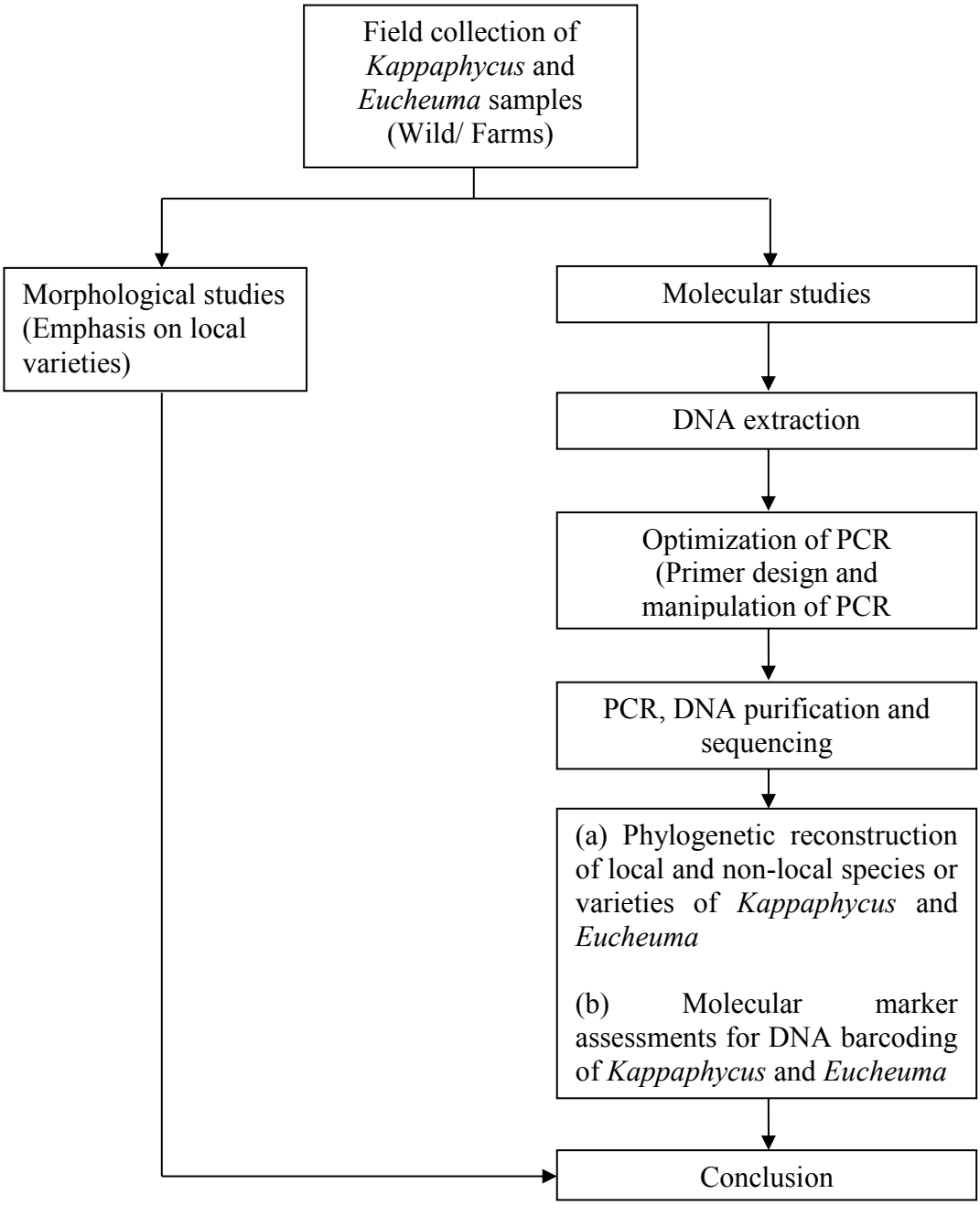

Figure 1.1: Flow chart showing the proposed research approach for this study

CHAPTER 2: LITERATURE REVIEW

2.1 Rhodophyta

Rhodophytes, more commonly known as red algae, are one of the most primitive eukaryotic algal groups, with a conservative estimate of 2500-6000 species in about 680 genera (Ragan et al. 1994; Woelkerling 1990). They are characterized by: (1) the absence of flagella, basal bodies and centrioles; (2) the presence of floridean starch as storage; (3) the presence of phycobiliprotein pigments and chlorophyll *a* only; (4) the lack of external endoplasmic reticulum within choloroplasts and (5) unstacked thylakoids (Adl et al. 2005; Freshwater et al. 1994; Woelkerling 1990). These algae are mostly multicellular, macroscopic, and predominantly occur in marine environments. Red algae undergo sexual reproduction and mostly exhibit a triphasic alternation of generations- two sporophyte generations and one gametophyte generation (Kohlmeyer 1975).

The taxa of red algae occupy a broad range of habitats, ranging from tropical, temperate to cold-water localities (Lüning 1990), playing an essential role as primary producers in food webs. Coralline red algae in the order Corallinales, which deposit calcium carbonate, are also ecologically important in the development and sustenance of coral reefs and their biota. Apart from being consumed as condiments and delicacies, certain rhodophytes are of significant commercial value because of the hydrocolloids (mainly agar and carrageenan) that they produce, which are to date important commodities for the seaweed industry (Bindu and Levine 2010; Bixler 1996; Bixler and Porse 2010; Phang et al. 2010).

The taxonomic status of rhodophytes has generally been convoluted and inconsistent, where life cycles, morphological and chemical characteristics do not always coincide with phylogenetic inferences (Ragan et al. 1994). Rhodophyta was

traditionally divided into two Classes- Bangiophyceae and Florideophyceae, which were subsequently revised into one class, Rhodophyceae with two subclasses Bangiophycidae and Florideophycidae. Members of Bangiophycidae were redefined based on organelle ultrastructure and mode of spore formation, whereas those of Florideophycidae emphasized on pit connections (Freshwater et al. 1994). Increased utilization of nuclear and plastid-encoded molecular markers at that time has supported the monophyly of Florideophycidae, but inferred polyphyly in Bangiophycidae (Freshwater et al. 1994; Ragan et al. 1994). Adl and co-workers (2005) subsequently proposed the classification of rhodophytes under Archaeplastida, along with green algae, land plants and glaucophytes. The new hierarchical system designates rhodophytes under Rhodophyceae, without employing formal taxonomic ranks i.e. “class”, “subclass” etc. for increased utility (Adl et al. 2005). This system has since then received mixed reviews, where some research coincides with the proposed rhodophyte taxonomic structure (Burki et al. 2009; Butler et al. 2007; Chan et al. 2011), while some do not (Kim and Graham 2008; Nozaki et al. 2009).

Yoon and co-workers (2006) proposed a different classification system where Rhodophyta is divided into two subphylums- Cyanidiophytina and Rhodophytina. Taxonomic ranks were re-introduced: Cyanidiophytina with one class i.e. Cyanidiophyceae; Rhodophytina with six classes i.e. Bangiophyceae, Compsopogonophyceae, Florideophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae. Even so, the taxonomic position of Rhodophyta has yet to reach a consensus as a result of limited studies above ordinal level.

2.2 *Kappaphycus* and *Eucheuma*

Members of the order Gigartinales and the family Solieriaceae, *Kappaphycus* Doty and *Eucheuma* J. Agardh are two of the most important carrageenan producers in the world. The taxonomic classifications of these red seaweeds are shown in Figure 1.2. These two carrageenophytes thrive mostly in tropical regions of the world.

The genus *Eucheuma* was established by J. Agardh in the year 1847, and *Kappaphycus* much later by Maxwell Doty in 1985. The original, generic morphological characteristics described for *Eucheuma* at that time include macroscopically the relatively coarse, generally bushy, rigid nature of the thalli and microscopically the presence of a rhizoidal medullary core, rotund medullary cells and a cortex of radiating filaments of elongated, smaller cells (Agardh 1847, 1852, 1892; Doty 1988; Harvey 1853). These characters still serve as a basis for taxonomic identification today.

Taxonomists J. Agardh (1847, 1852, 1876, 1892), Doty (1973, 1985, 1987, 1988; Doty and Alvarez 1975; Doty and Norris 1985), Schmitz (1985), Weber-van Bosse (1913, 1926, 1928) and Yamada (1936) contributed greatly to the progression of the genus *Eucheuma*, with many constituting species being commercial important. Earlier microscopic studies only allowed preliminary observations on specimens; however, the improvements in terms of microscopy technologies enabled more detailed studies on the reproductive structures of *Eucheuma* after the 1950s. Subsequent identification and description of new species of *Eucheuma* relied heavily on morphological attributes, at least until the finding that different types of carrageenan were actually produced by different eucheumatoids (samples showing characteristics of *Eucheuma*). Based on this knowledge, Doty (1988) erected the genus *Kappaphycus*, which is essentially composed of species producing *kappa*-carrageenan, including the then newly domesticated

Kappaphycus alvarezii (*Eucheuma alvarezii*) aimed at commercial carrageenan production.

Apart from introducing the genus *Kappaphycus*, Doty (1988) divided *Eucheuma* into three sections, namely *Eucheuma* section *Eucheuma*, *Eucheuma* section *Gelatiformia*, and *Eucheuma* section *Anaxiferae*. Each section was differentiated from one another mainly by the branching patterns, characteristics of the axial core, cystocarp positions and carrageenan types. Comprehensive keys to differentiating *Eucheuma*, as well as species resembling *Eucheuma,* were constructed (Doty 1988; Cheney 1988).

As of now, the Algaebase (http://www.algaebase.org/) database (Guiry and Guiry 2013) records five species of *Kappaphycus*, namely *K. alvarezii* (Doty) Doty ex P. C. Silva, *K. cottonii* (Weber-van Bosse) Doty ex P. C. Silva, *K. inermis* (F. Schmitz) Doty ex H. D. Nguyen & Q. N. Huynh, *K. procrusteanus* (Kraft) Doty and *K. striatus* (F. Schmitz) Doty ex P. C. Silva and a variety *K. alvarezii* var. *tambalang* (Doty). This variety was reported as not being validly described (Guiry and Guiry 2013). *Kappaphycus alvarezii* (originally named *Eucheuma alvarezii*) was first discovered in the Creagh Reef which is south of Semporna, Sabah, Malaysia; and was designated type species of the genus *Kappaphycus*.

On the other hand, 37 *Eucheuma* species are regarded as taxonomically accepted under Algaebase. These include *Eucheuma adhaerens* Weber-van Bosse, *E. alvarezii* var. *ajakii-assii* Doty, *E. amakusaense* Okamura, *E. arnoldii* Weber-van Bosse, *E. arnoldii* var. *alcyonida* Kraft, *E. cartilagineum* Dewitz, *E. cervicorne* Weber-van Bosse, *E. chondriforme* J. Agardh, *E. crassum* Zanardini, *E. crustiforme* Weber-van Bosse, *E. deformans* P. W. Gabrielson & Kraft, *E. denticulatum* (N. L. Burman) F. S. Collins & Hervey, *E. dichotomum* Weber-van Bosse, *E. edule* (Kützing) Weber-van Bosse, *E. edule f. majus* Weber-van Bosse, *E. horizontale* Weber-van Bosse, *E. horridum* J.

Agardh, *E. horridum f. radicans* Børgesen, *E. isiforme* (C. Agardh) J. Agardh, *E. isiforme* var. *denudatum* D. P. Cheney, *E. johnstonii* Setchell & Gardner, *E. jugatum* J. Agardh, *E. kraftianum* Doty, *E. leeuwenii* Weber-van Bosse, *E. nodulosum* Areschoug, *E. nudum* J. Agardh, *E. odontophorum* Børgesen, *E. odontophorum* var. *mauritianum* (Børgesen) Doty ex P. C. Silva, *E. perplexum* Doty, *E. platycladum* F. Schmitz, *E. serra* (J. Agardh) J. Agardh, *E. simplex* Weber-van Bosse, *E. sonderi* Harvey, *E. uncinatum* Setchell & Gardner, *E. vermiculare* Weber-van Bosse and *E. xishaensis* Kuang Mei & Xia. The type species of *Eucheuma* is *E. spinosum* J. Agardh (synonymous to the currently accepted *E. denticulatum* (N. L. Burman) F. S. Collins & Hervey.

Despite the availability of a dichotomous key for species identification of *Kappaphycus* and *Eucheuma*, recent research involving molecular taxonomy has revealed that morphological attributes are not always accurate in species identification, caused mainly by the morphologically plastic nature of these red seaweeds (Conklin et al. 2009; Dang et al. 2008; Zuccarello et al. 2006). The taxonomic elucidation of *Kappaphycus* and *Eucheuma* is still an ongoing effort.

Phylum	Rhodophyta
Subphylum	Rhodophytina
Class	Florideophyceae
Subclass	Rhodymeniophycidae
Order	Gigartinales
Family	Solieriaceae

Figure 1.2: Taxonomic classification of *Kappaphycus* Doty and *Eucheuma* J. Agardh based on the classification system by Yoon et al. (2006)

2.2.1 Morphology

Kappaphycus seaweeds are generally large, capable of growing up to 1-2 meters in size, and produce *kappa* carrageenan. Morphological descriptions of *Kappaphycus* were somewhat confusing due to the wide range of morphological and color variations, even within the same species. The loss of size and structure in herbarium specimens has also been regarded as a challenge in morphological comparisons among species or even genera. Still, despite the morphological plasticity, all fresh *Kappaphycus* seaweeds, regardless of the gametophytic or sporophytic stage, are multiaxial, with generally fleshy and smooth thalli. Thalli are mostly cylindrical, although some may become compressed or clumped when exposed to varying environmental pressures. Branching is indeterminate, and may range from irregular, unilateral to orderly depending on species. Terminal branches may range from slender and attenuated to dichotomous or

trichotomous. Wild specimens, if undamaged, often arise from a discoid, crustose holdfast (Doty 1985; Doty and Norris 1985). Cystocarps of *Kappaphycus* tend to grow on the main segments of the main axes as swollen protrusions, and are internally composed of a relatively large spherical fusion cell that radiates gonimoblast filaments (Doty 1988). There are no lateral outgrowths associated with cystocarps (Doty 1988). It is believed that there are no obvious morphological differences between non-fertile tetrasporophytic and gametophytic *Kappaphycus* seaweeds. Tetrasporangia are zonate, whereas carpospores and tetraspores (seriately divided) are generally similar.

Microscopically, the inner and outer cortexes are apparent, where the latter is composed of pigmented, elongated cells arranged in a radial fashion. Cells within the inner cortex are generally radially elongated to isodiametric, and become larger and more spherical towards the core. Pit-plug connections are present among neighboring cells. Medullary cells are generally isodiametric in transections, where primary cells often have thylles (yeast-like buddings from large medullary or inner cortical cells and persist as small, somewhat elongated cells among large ones, especially in the central axial region) occurring individually or in irregular clusters. Within the apical regions, the core consists of filaments of cells (longitudinal view) which become narrower when nearing the center of the core. These cells become less conspicuous away from the apical tips, replaced successively by larger, irregularly positioned cells; surrounded by cells of smaller but random sizes towards the base (Doty 1985).

Eucheuma can be distinguished from *Kappaphycus* based on the production of *iota* or *beta*-carrageenan and some distinctive morphological characters. However, similar to *Kappaphycus*, morphological plasticity has rendered morphological descriptions taxing, especially so when the number of taxa is significantly larger. Morphologically, *Eucheuma* exhibits cylindrical, fleshy to brittle fronds with simple spines which are arranged in a generally orderly pinnate or pectinate fashion from the

main axis or branches (Doty 1988). Branches predominantly arise through spines becoming indeterminate. Cystocarps may be found on laterals or on main axes depending on species, and the internal anatomy was reported to be generally similar to that of *Kappaphycus* (Doty 1988; Doty and Norris 1985). Tetrasporangia are again zonate.

Distinctive microscopic traits include the abundance of rhizoidal filaments in spines, absence of hyphal structures arising from thylles, and the presence of a central medullary rhizoidal axial strand (Doty 1988). Considering the extensive range of vegetative tendencies, the genus *Eucheuma*, as aforementioned, has been divided into three sections, namely *Eucheuma* section *Eucheuma* Doty, *Eucheuma* section *Gelatiformia* Weber-Van Bosse and *Eucheuma* section *Anaxiferae* Doty and Norris, each with unique morphological attributes (Doty and Norris, 1985; Doty 1988).

According to Doty (1988), members of *Eucheuma* section *Eucheuma* display cylindrical fronds and simple spines, with basal diameters less than their axis thickness. Spines occur in regular pairs or whorls first, becoming more scattered farther away from the base. Branches generally form whorls, but may range from regularly opposite, pectinate, to irregular. Members within this section produce *iota*-carrageenan. In terms of reproductive structures, cystocarps are associated with laterals, often with a single spine beyond each cystocarp. Microscopically, the axial cores are rhizoidal and cylindrical.

Eucheuma section *Gelatiformia* represents species with compressed fronds, simple spines and basal diameters equal to that of the axis. Spines occur in rows, marginally first and later occurring dorsally and ventrally on flatter faces, or scattered altogether. Branches are mostly marginal, pinnate to irregular, but not pectinate. Carrageenan types may range from *beta* to *iota*. Cystocarps often occur on laterals, with

no spines or up to several spines associated with the sides of cystocarps. Members of this section often exhibit flattened, tortuous hyphal axial cores (Doty 1988).

Doty and Norris (1985) and Doty (1988) have described members of *Eucheuma* section *Anaxiferae* as having cylindrical or dorsiventral fronds bearing compound spines. Distribution of spines is often in whorls and scattered in various arrangements and intensities. Branching is often opposite, whorled or irregular. Members of this section produce *iota*-carrageenan. Cystocarps occur on main axes and are not associated with any spines.

There is little change in the classification of *Eucheuma* since that of Doty (1988), but the taxonomy of this genus is still poorly understood at this juncture, and far from complete.

2.2.2 Cultivation

Although initial production of carrageenan relied heavily on the collection of *Kappaphycus* and *Eucheuma* from wild populations, increasing demands and dwindling natural populations have led to their introduction of cultivation methods through the collaboration between Dr. Maxwell Doty and Marine Colloids in 1971 (Ask and Azanza 2002; Ask et al. 2003; Bindu and Levine 2010; Doty and Norris 1985; Neish 2003; Santelices 1999; Trono 1992). *Kappaphycus alvarezii*, *K. striatus* and *Eucheuma denticulatum* were successfully cultivated on a massive scale, and were subsequently introduced to other countries for commercial purposes, leading to an upward spiral in carrageenan production ever since (Ask and Azanza 2002; Ask et al. 2003; Ask et al. 2001; Munoz et al. 2004; Neish 2003).

Cultivation of *Kappaphycus* and *Eucheuma* are basically done using three simple and economical methods: (1) Fixed off-bottom monoline method; (2) monoline or longline method and (3) floating rafts method (Neish 2003; Sulu et al. 2003; Trono

1992). These three methods essentially involve the usage of long nylon lines on which *Kappaphycus* or *Eucheuma* thalli are tied e.g. using the *tie-tie* method (approximately 30 cm to 1 m apart) for vegetative spawning.

The fixed off-bottom monoline method as depicted in Figure 2.1 involves the drilling and insertion of long mangrove stakes into the substratum at sites deemed suitable for cultivation i.e. appropriate water depth and currents, nutrient rich seawater, ample sunlight etc. The stakes should be approximately one meter between rows, and can be up to 1000 m between columns, where nylon monolines are stretched and tied (in between column mangrove stakes). Choice of monoline lengths varies, depending on size of farm, economic feasibility, available workforce and expected crop production. The distance of the monoline to the seabed is generally determined based on the water depth during low tides (Neish 2003; Santelices 1999; Trono 1992) .

The monoline or longline methods (Figure 2.2) remove the need for mangrove stakes, and also offer better flexibility as they are suitable for areas with uneven seabed as well as deeper waters. Conventionally, bamboos which are arranged at intervals of approximately 5 m are used as floating devices that keep the tied nylon lines (30 cm apart from one another) in place. Again the length of the nylon filaments and the number of lines to be tied on one bamboo may vary depending on several factors as aforementioned. The bamboos are then securely anchored to the substratum by means of wooden or metal spikes at both ends (Neish 2003; Trono 1992). Current trends in cultivation see the replacement of bamboo with floats, which are tied at 3-5 m intervals along the monoline or longline.

The floating raft method (Figure 2.3) is generally the least used (depending on countries), possibly due to the higher costs and manpower needed to construct rafts, but they are suitable in deeper waters, and also offer mobility (for repositioning or removal

during bad weather) when the anchoring stakes are removed. Principally a bamboo "frame" of approximately 3 x 3 meters or 4 x 4 meters with polypropylene ropes tied in parallel at intervals of 10-15 cm. The raft is anchored to the seabed so as to keep the bamboo rafts at an approximate 50 cm below water surface. Additionally, the bamboo mainframe can also be modified with an inclusion of fishing nets to avoid herbivory (Johnson and Gopakumar 2011; McHugh 2003b).

Upon setting up of the cultivation system, minimal maintenance is required to ensure sustainable growth of the seaweed crops. This includes removal of epiphytes or other marine grazers, replacing poorly growing or lost cultivars, and of course, the repair or replacement of damaged bamboo, stakes or lines (McHugh 2003b; Trono 1992; Neish 2003). It is often advisable to alternate farming areas between each planting season to avoid loss of site fertility. Harvesting of *Kappaphycus* and *Eucheuma* is fairly straightforward, where the *tie-ties* are cut, and the entire seaweed collected. This is usually done manually by farmers, or on a larger scale by specially-designed harvester boats.

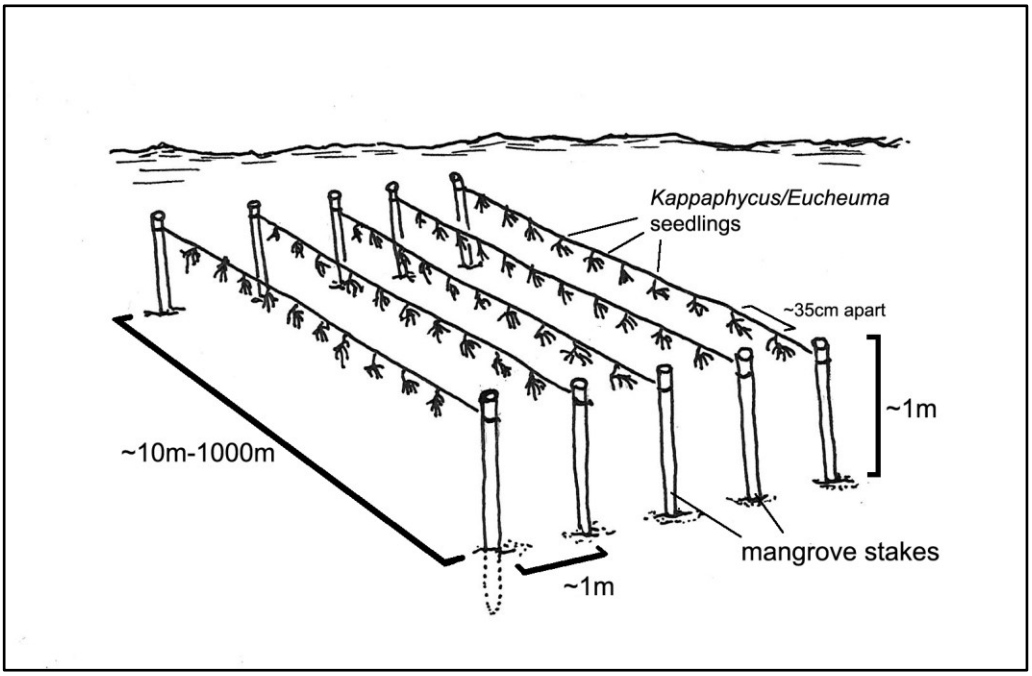

Figure 2.1: Fixed Off-Bottom Monoline Cultivation Method. Suggested materials and parameters may vary depending on environmental factors. Picture not drawn to scale.

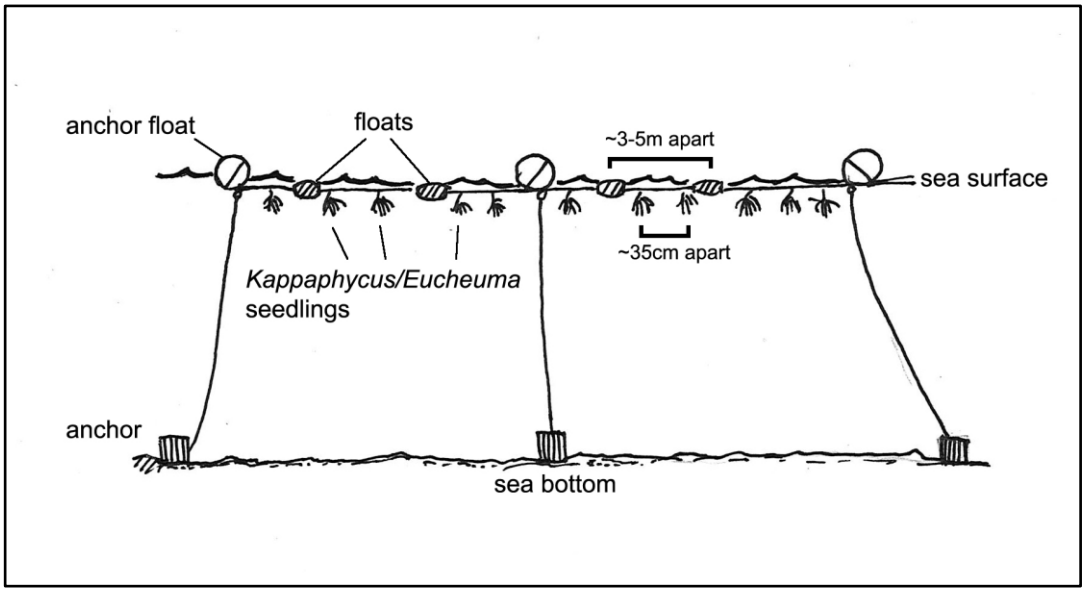

Figure 2.2: Monoline or Longline Cultivation Method. Suggested materials and parameters may vary depending on environmental factors. Picture not drawn to scale.

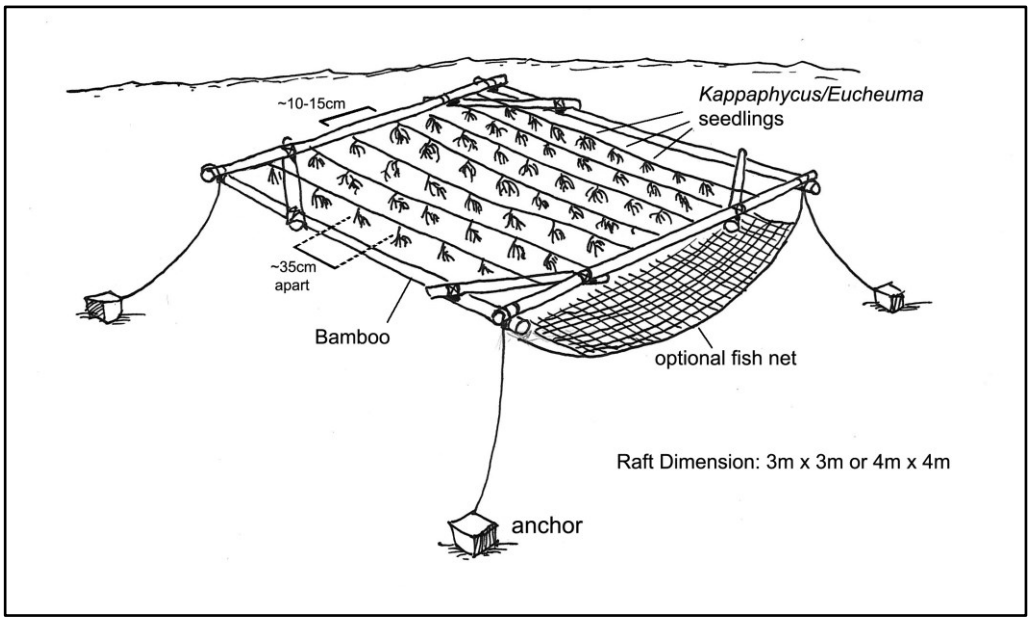

Figure 2.3: Floating Raft Cultivation Method. Suggested materials and parameters may vary depending on environmental factors. Picture not drawn to scale.

2.2.3 Processing

In order for carrageenan extraction to be conducted, the harvested *Kappaphycus* and *Eucheuma* seaweeds need to be cleaned of epiphytes, marine organisms and other foreign materials prior to drying. Although *Kappaphycus* produces *kappa*-carrageenan whereas *Eucheuma* produces *iota*-carrageenan, the drying methods are similar, so long as the two different carrageenophytes are not mixed together. Harvested seaweeds are usually evenly spread out and sun-dried on farm platforms until the crops are bleached, with less than 40% moisture (McHugh 2003b; Neish 2003; Trono 1992). For budget platforms, waterproof PVC canvases are often used to cover the seaweeds during poor weather conditions; higher-end platforms may incorporate enclosed drying rooms for better desiccation and weather-proofing.

According to Trono et al. (1992), *Kappaphycus* and *Eucheuma* are exported in four forms, as (1) dried seaweed; (2) alkali-treated chips; (3) semi-processed powder or (4) pure carrageenan, of which the latter two are of higher popularity and demand. During the 1970s, pure carrageenan was largely produced as gelling agents for canned meat pet foods, but eventually replaced by semi-refined extracts which are significantly cheaper and easier to produce (Bixler 1996; Bixler and Porse 2010). Advances in processing technology by processing factories have also enabled proper sterilization techniques for the mentioned semi-refined carrageenan (SRC), leading to more stringent quality control. Market trends have since then changed towards the production of higher quality human food-grade SRC- Processed *Eucheuma* Seaweed (PES) or Philippine Natural Grade (PNG in the Philippines) (both coded E-407a), which has been labeled as different from refined carrageenan (coded E-407) by the European Commision and the FAO/WHO Codex Alimentarius, thus requiring different ingredient labels (Bixler and Porse 2010; McHugh 2003b).

The processing methods for the extraction of refined or semi-refined carrageenan (Figure 2.4) from dried seaweed are generally well known, as follows:

(a) Refined carrageenan

Upon complete drying (when sold to processors), seaweeds are subjected to washing to remove excessive sand, salt crystals as well as other foreign materials. The cleaned product is subsequently soaked in alkali-treated (sodium hydroxide) water before being heated for a few hours. The alkali treatment results in chemical changes within the seaweeds, forming more 3, 6-anhydrogalactose units which increases gel rigidity (McHugh 2003a, 2003b; Mendoza et al. 2002; Neish 2003; Yu et al. 2002). Residual seaweed is removed via centrifugation or coarse filtration, and the resulting solution further filtered using fine filtration, producing a 1-2% carrageenan solution which can be concentrated by vaccum distillation or ultrafiltration (McHugh 2003b). An alcohol precipitation or gel pressing method then ensues in order to obtain carrageenan in solid form, with the latter only applicable to *kappa*-carrageenan.

The alcohol precipitation method involves constant soaking of the carrageenan solution until coagulated precipitates are formed, which are then filtered out using centrifugation or fine filtration. Further dehydration by alcohol is applied to the resulting coagulum, followed by milling to a suitable size before the refined carrageenan can be packeted and sold (McHugh 2003b; Neish 2003; McHugh 2003a).

Kappa-carrageenan has the tendency to form potassium salts when exposed to potassium ions. The gel method takes advantage of this particular chemical property, where the carrageenan solution is fine-filtered using potassium chloride solution. This is done several times, and eventually pressed to remove excessive water or liquids before being frozen and then thawed (also to remove water). Again washed with potassium

chloride, the resulting gel-like materials are heat-dried and milled (McHugh 2003b; Neish 2003; McHugh 2003a).

(b) Semi-refined carrageenan

SRC is produced using the potassium chloride extraction method, but is applied directly to the dried seaweeds (does not involve the extraction of the carrageenan solution). The production of SRC is considerably cheaper than that of refined carrageenan since it does not require the use of alcohol, alcohol distillator, freezers etc. *Kappaphycus* seaweeds are soaked in potassium hydroxide and then heated for several hours to increase gel strength and also solubilize undesired entities e.g. protein, salts, carbohydrate etc. The resulting seaweeds, now somewhat internally concentrated with carrageenan, are thoroughly washed with water to remove foreign materials, then again sun-dried for one or two days before being milled into SRC powder and marketed (McHugh 2003b; Neish 2003; Trono 1992; McHugh 2003a). Semi-refined carrageenans are not free of microorganisms and thus require sterilization prior to utilization in products for canned pet food. In order to produce SRC with lower bacterial counts, an additional bleaching step and stricter drying protocols are required for human grade carrageenan (Bixler and Porse 2010; McHugh 2003b; Neish 2003; McHugh 2003a).

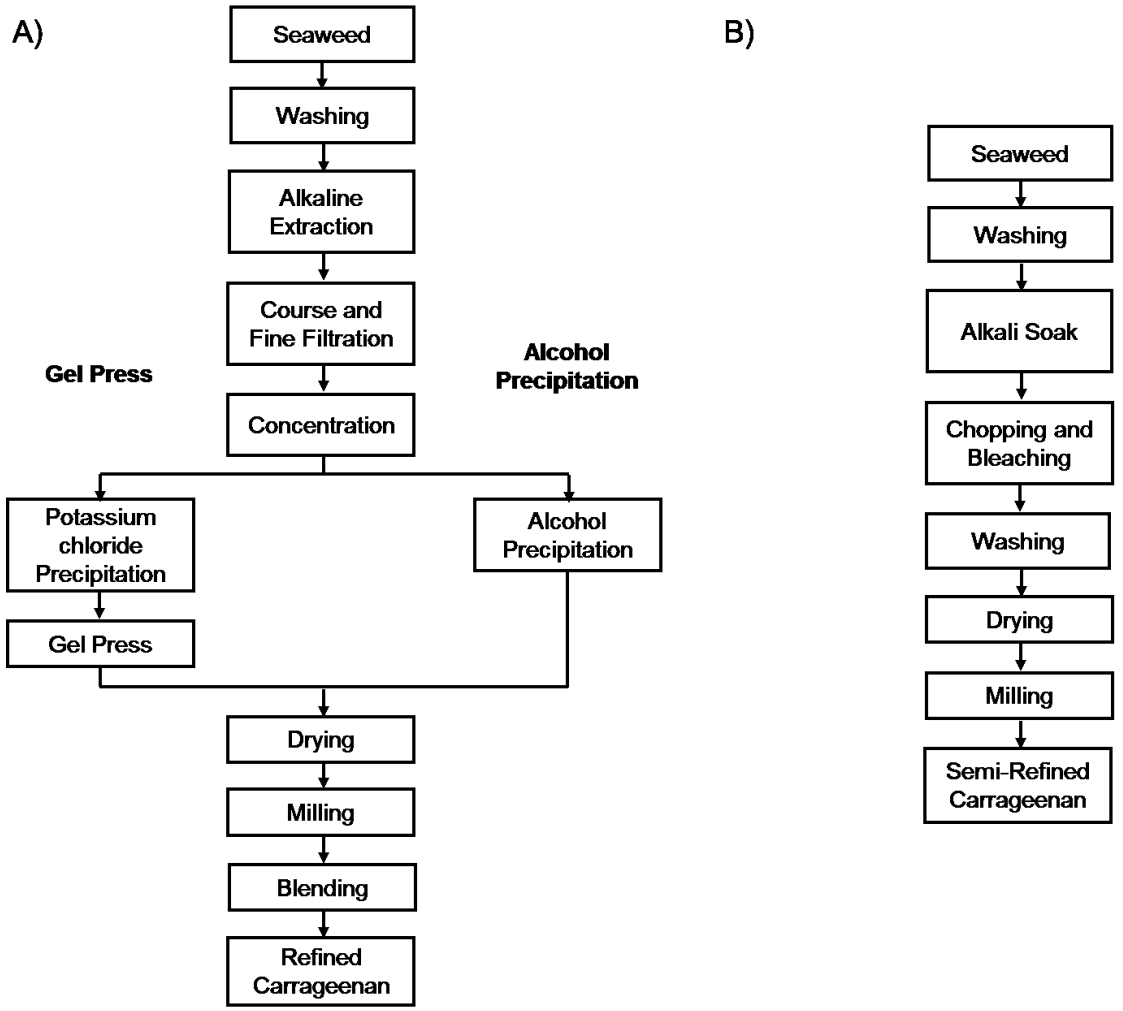

Figure 2.4: Flow chart showing the preparation methods for Refined Carrageenan (A) and Semi-Refined Carrageenan (B). Adopted from Bixler (1996) and Porse (1998).

2.2.4 Economic Importance

Kappaphycus and *Eucheuma* are commercially important because of the carrageenan they produce. Although there are various types of carrageenan e.g. *alpha*, *beta*, *kappa*, *iota*, *lambda*, *mu* and *nu* (Doty 1988; Phang et al. 2010), *kappa*- (from *Kappaphycus*) and *iota*-carrageenan (from *Eucheuma*) are by far the most widely marketed. Costing about 10.5 US dollars per kg, the sales value of 527 million US dollars was recorded for carrageenan in 2009, significantly higher than that of agar and alginate (Bixler and Porse 2010). This has generated profits not only for the hydrocolloid processors, but also the seaweed farmers. Although there is still much room for improvement, seaweed cultivation has offered job opportunities and income to the poor (Ask et al. 2003; Ask et al. 2001; Bindu and Levine 2010; Hurtado et al. 2001; Phang et al. 2010).

Kappa-carrageenan is characterized by the ability to form strong gels when exposed to potassium ions and can be separated from liquid by contraction (synaeresis); whereas *iota*-carrageenan forms soft gels when exposed to calcium ions and does not undergo synaeresis (McHugh 2003a). These attributes lead to gelling, thickening and emulsifying properties which are commercially important. Despite the predominance of *Kappaphycus* and *Eucheuma* cultivation in many parts of the world, the cultivation of *Eucheuma* and thus *iota*-carrageenan production is approximately one seventh that of *Kappaphycus*, and can be ascribed to the lower growth rates and also market demands (Bixler and Porse 2010; Neish 2003; Zuccarello et al. 2006).

Carrageenan, owing to its unique properties, is extensively used in the food (ice creams, desserts, fruit juices etc.) and cosmetics industries (toothpaste, shampoos, lubricants etc.). Apart from that, carrageenan has also been tested for medical uses as topical microbicides for sexually transmitted diseases (Buck et al. 2006; Roberts et al.

2007) and also cell immobilization (Gòdia 1987; Moon and Parulekar 1991; Wang and Hettwer 1982). Although fairly new, exploitation of potential immunostimulatory and antitumor activity (Yuan and Song 2005; Yuan et al. 2010), and bioethanol production (Meinita et al. 2011), if feasible, would undoubtedly increase the economic importance of *Kappaphycus* and *Eucheuma*.

2.2.5 Controversies

The biggest disputes on *Kappaphycus* and *Eucheuma*, specifically the carrageenan they produce, are associated with health issues, which are still unresolved. Concerns were sparked when Tobacman (2001) reported a link between degraded carrageenan i.e. poligeenan and the development of ulceration and gastro-intestinal cancer in animal models, leading to a much stricter control of poligeenan in food additives, particularly those in infant formulas. The amount of poligeenan allowed in carrageenan was limited to 5% by a scientific committee representing the European Commission. Subsequent research has shown that carrageenan induces inflammation in human intestinal epithelial cells *in vitro* through a distinct Bc110 pathway (Borthakur et al. 2006). There have been few updates on the negative effects of carrageenan ever since, although price increments have been substantial due to the regulatory order. More health-based research is required in order to investigate the drawbacks of these otherwise largely popular carrageenophytes.

Apart from arguments on food safety, the potential bioinvasive effects of *Kappaphycus* and *Eucheuma* on local coral reefs and their inhabitants are also a major concern, considering the widespread introduction of mainly *K. alvarezii* for commercial farming in many countries, a lot of which were properly documented (Bixler and Porse 2010; Doty 1985, 1988; Doty and Norris 1985; Gerung and Ohno 1997; Halling et al. 2012; Hung et al. 2008; Munoz et al. 2004; Neish 2003; Phang et al. 2010; Russell 1983;

Trono 1992; Zuccarello et al. 2006; Pickering 2006). Reports has demonstrated that *K. alvarezii* have successfully invaded and become established on both live and dead corals in the Gulf of Mannar, India, eliminating especially natural populations of *Acropora* and *Turbinaria* due to fouling and smothering effects (Chandrasekaran et al. 2008; Kamalakannan et al. 2010). These reports have refuted previous claims that *Kappaphycus* were coral-friendly and safe for mass cultivation in wild areas (Janodia et al. 2006; Mandal et al. 2010; Russell 1983); safety precautions and countermeasures are required, especially for countries with introduced strains of *Kappaphycus* or *Eucheuma* in order to avoid imminent destruction of local habitats and their ecology.

2.3 Molecular taxonomy, phylogenetics and its implications

The extensive morphological plasticity and the paucity of clear distinguishing characters of *Kappaphycus* and *Eucheuma* are major setbacks to proper establishment of a taxonomic scheme for these red seaweeds. Despite several alterations in terms of classification back in the 1980s, the overall taxonomic status of *Kappaphycus* and *Eucheuma* remains poorly studied (Bixler and Porse 2010; Conklin et al. 2009; Dang et al. 2008; Doty 1988; Doty and Norris 1985; McHugh 2003b; Neish 2003; Phang et al. 2010; Vairappan 2006; Zuccarello et al. 2006).

The application of molecular phylogenetics by Zuccarello et al. (2006) on *Kappaphycus* and *Eucheuma* has brought about promising results which will provide data for the revision of these carrageenophyte taxa. Molecular taxonomy or molecular phylogenetics essentially involves analyses used to estimate heredity and relationships between organisms based on molecular differences among DNA or amino acid sequences. Recent phylogenetic studies involve DNA rather than protein sequences due to the ease of amplification and higher throughputs. All living organisms have DNA, RNA and proteins, which can be used as a basis for phylogenetic comparsion and

inferences. DNA accumulates mutations over time, with non-protein coding regions often mutating faster than protein coding regions. This is because proteins require specific conformational structures in order to function normally, or will otherwise be deleterious to the general wellbeing of an organism; therefore the mutation rates observed in protein coding regions are often more conserved, as a single change in DNA nucleotide may alter the entire structure of the encoded protein, or cause an entire frameshift of amino acid translation, often resulting in death of the host organism (Page and Holmes 1998). However, due to the degenerate properties of the genetic code i.e. different codons may code for the same amino acid, certain mutations (usually at the 3rd nucleotide position of each codon triplet), do not affect the final 3D structure of the encoded protein, and are thus termed silent mutations. These mutations will accumulate with time. Molecular systematics employs dedicated bioinformatics programs that detect these mutations or alterations between homologous sequences, and from there, estimate organisms' evolutionary relationships and present them via phylogenetic trees.

Using the non-protein coding mitochondrial encoded *cox*2-3 spacer and plastid encoded RuBisCO spacer developed earlier for other Rhodophytes (Zuccarello et al. 1999; Zuccarello et al. 1999b), a preliminary, but informative phylogenetic interpretation of *Kappaphycus* and *Eucheuma* collected from various part of the world was derived. Zuccarello and co-workers (2006) highlighted several important findings: (1) Both the *cox*2-3 spacer and RuBisCO spacer were capable of phylogenetically delineating members of the *Kappaphycus* and *Eucheuma* genera, with the former offering higher resolving power than the latter; (2) There is a clear genetic difference between *Kappaphycus alvarezii* and *K. striatus* as well as *Eucheuma denticulatum*; (3) *K. alvarezii* from Hawaii and Africa appeared to be a different genetic lineage from the main *K. alvarezii* haplotype from around the globe; (4) *Eucheuma denticulatum* from Africa was also genetically distinct from the main haplotype; (5) *Eucheuma* may be

paraphyletic; and (6) The *cox*2-3 spacer and RuBisCO genetic markers were not capable of distinguishing *Kappaphycus* and *Eucheuma* morphotypes below species level. This study has become the basis of many studies that followed.

Using the mitochondrial *cox*2-3 spacer, which was shown to be better at resolving the phylogeny of *Kappaphycus* and *Eucheuma* (Zuccarello et al. 2006), Conklin and co-workers (2009) have, along with the newly introduced partial nuclear 28S rRNA, partial plastid 23S rRNA and mitochondrial 5' *cox*1 genetic markers; genetically and biogeographically archived *Kappaphycus* and *Eucheuma* seaweeds in Hawaii, enabling them to monitor and prepare for potential bioinvasions such as those reported in India (Chandrasekaran et al. 2008; Kamalakannan et al. 2010). Apart from the phylogeny, haplotype networks were generated for both *Kappaphycus* and *Eucheuma*, and are useful in identifying and selecting good strains for future cultivation (Halling et al. 2012; Zuccarello et al. 2006). Nuclear encoded rDNA Internal Transcribed Spacer (ITS) (Zhao and He 2011), plastid URP1 (Ganzon-Fortes et al. 2011; Provan et al. 2004) and *rbc*L (Fredericq et al. 1999; Freshwater et al. 2010) genetic regions have also been used, albeit on a smaller scale, for phylogenetic delineation and species identification. Despite the numerous DNA markers used, the mitochondrial *cox*2-3 spacer is currently the most widely used, with its popularity ascribed to the short length and ease of amplification as well as the relatively large number of GenBank records (Halling et al. 2012; Zuccarello et al. 1999; Zuccarello et al. 2006).

Although several phylogenetic analyses have already been carried out, most are localized and do not cover *Kappaphycus* and *Eucheuma* from Southeast Asia. This will lead to an oversimplification of genetic diversity of these red algae since the countries within that region, particular those encompassed by the Coral Triangle, i.e. Indonesia, Malaysia and the Philippines, are supposedly richest in terms of biodiversity in the world. The application of molecular taxonomy is thus significant in this region, not only

for phylogenetic reconstruction, but also for genetic diversity studies. Additionally, molecular phylogeny may also prove useful in elucidating the extensive, uncontrolled and confusing usage of local as well as commercial names pertaining to *Kappaphycus* and *Eucheuma*.

2.4 DNA barcoding

2.4.1 Origin of DNA barcoding and its applications

Biodiversity has gained much interest since the 1990s in many fields including ecology, taxonomy, agriculture etc., where species identification is becoming increasingly important. In views of the advances in molecular technology and molecular systematics, Herbert et al. (2003a; 2003b) proposed the usage of a short 648 bp region of the mitochondrial *cox*1 gene as a universal DNA marker aimed at providing fast and accurate identification of organisms. This technique was termed "DNA barcoding". Considering the universality of mitochondria in eukaryotic organisms, the scope of DNA barcoding can be impressively extensive and the method has since its introduction become widely applied in the scientific world. The Barcode of Life Data System (BOLD) is evidently the largest initiative in DNA barcoding, attempting to genetically barcode every single species on the surface of Earth, and establish a DNA barcode library for referencing (Jinbo et al. 2011; Ratnasingham and Hebert 2007; Sarkar and Trizna 2011; Wong et al. 2011).

Simple, cost-effective, rapid and accurate, DNA barcoding has provided both seasoned "conventional" taxonomists and non-experts a means to correctly identify specimens, a technique foreseen, and long awaited (Blaxter 2003; Busse et al. 1996; Jinbo et al. 2011). The DNA sequences extracted from old, small or damaged herbaria (usually type specimens) can be used as supplementary data for taxonomic description or even establishment; whereas DNA barcodes generated for recently described species

would aid in subsequent identification or systematic efforts. Apart from the taxonomical aspects, the employment of DNA barcoding also bring about useful applications, such as the identification of cryptic host specificity (Smith et al. 2006; Smith et al. 2007; Jurado-Rivera et al. 2009), investigating trophic relationships (Clare et al. 2009; Mathesona et al. 2008; Weber and Lundgren 2009), detecting mislabeling and the illegal trade of products (Eaton et al. 2009; Lowenstein et al. 2009) as well as identification of specimens at various developmental stages which may not have distinctive, identifiable morphological characters (Pieterse et al. 2010; Emery et al. 2009; Malumphy et al. 2009), just to name a few.

DNA barcoding on red algae was started by Saunders (2005) and Robba and co-workers (2006), a few years after its debut by Hebert and co-workers (2003). Still, genetic variability between a few mitochondrial, nuclear or plastid-encoded DNA markers have been compared and discussed well before that, some of which are even recommended as potential DNA barcodes today. Initial efforts on red algal systematics were based on the Internal Transcribed Spacer (ITS) developed for fungi which encompasses the ITS1, 5.8S rDNA and the ITS2 regions (Goff et al. 2004; White et al. 1990), in which, despite its high genetic variation and ease of amplification, issues associated with alignment and intraspecific species identification has rendered it less feasible for genetic studies. Similarly, studies using nuclear-derived markers i.e. 18S rDNA have also revealed relatively low genetic divergence as compared to those of plastid origins (Ragan et al. 1994; Olson et al. 2004). Although many molecular markers were developed for molecular systematics, in general plastid and mitochondrial-encoded molecular markers were often recommended as good DNA barcodes for rhodophytes (Freshwater et al. 1994; Geraldino et al. 2006; Kim et al. 2010; Le Gall and Saunders 2010; Robba et al. 2006; Rueness 2010; Saunders 2005; Saunders 2008, 2009; Zuccarello and West 2011). This was supported by a recent, large-scale survey on

rhodophyte biodiversity in Hawaii (Sherwood et al. 2010), which employed the use of the mitochondrial *cox*1, nuclear LSU (28S) and plastid UPA (partial 23S), where it was demonstrated that the more conserved nuclear-encoded LSU was not as phylogenetically informative as the other two markers.

Recent efforts on DNA barcoding of red algae are more focused on the elucidation, and if needed, revision of systematics over broad taxa as well as construction of DNA libraries for future reference (Clarkston and Saunders 2010; Freshwater et al. 2010; Geraldino et al. 2006; Kim et al. 2010; Le Gall and Saunders 2010; Saunders 2008; Saunders 2009). These studies have provided a means of resolving taxonomic uncertainties often caused by morphological limitations. DNA barcoding has also been applied to biodiversity surveys and biomonitoring efforts (Carlile and Sherwood 2013; Conklin et al. 2009; Rueness 2010; Sherwood et al. 2010). With increasing records and better taxonomic classifications, the accuracy of DNA barcoding will improve, ultimately facilitating species identification, and other useful applications that follow e.g. studies on biodiversity, genetic diversity, host-pest relationships, phylogeography, biomonitoring, strain selection and so on.

2.4.2 Prerequisites for potential DNA barcodes and their accuracy

The mitochondrial encoded *cox*1 DNA barcode by Herbert and co-workers (2003a; 2003b; 2004) is not the sole barcode used. Studies on plants and fungi have shown that other genetic markers (*rbc*L and *mat*K for plants; and ITS for fungi respectively) appeared to be better at taxonomic resolution, and have thus been proposed and generally accepted as standard DNA barcodes for the associated taxa (Consortium for the Barcode of Life 2009; Seifert 2009; Seifert et al. 2007). Molecular markers are expected to fulfill numerous criteria prior to recommendation as potential DNA barcodes; criteria which include short length (<1.5 kb), universality, ease of amplification, preferably protein coding (ease of alignment), good resolution power and of course accurate species identification (Jinbo et al. 2011).

Accuracy of species identification is the most important aspect in DNA barcoding and is usually only apparent and assessable in large datasets which include reliable barcode libraries (Ekrem et al. 2007). Theoretically, the accuracy is determined by the extent of the gap between the interspecific divergence and the intraspecific variation of sequences within a particular dataset i.e. the larger the gap, the better the species identification (Hebert et al. 2004; Meyer and Paulay 2005). This gap is thus colloquially termed as a "Barcoding Gap" (Figure 2.5)- the difference between intraspecific and minimum congeneric distances (Meier et al. 2008). However, the success rates of species identification tend to decrease when there is an overlap between the inter- and intraspecific divergences (Figure 2.5) (Elias et al. 2007; Jinbo et al. 2011; Meyer and Paulay 2005; Moritz and Cicero 2004). This phenomenon may be caused by large genetic diversities, presence of recently diverged species, disagreement between molecular data and traditional species definitions, or incomplete lineage sorting of mitochondrial DNA (Avise and Walker 1999; Davis and Nixon 1992; DeSalle et al. 2012; Jinbo et al. 2011; Meyer and Paulay 2005); most, if not all of which could be

tackled with additional morphological data or molecular data from secondary markers (Hebert et al. 2003a; Meyer and Paulay 2005).

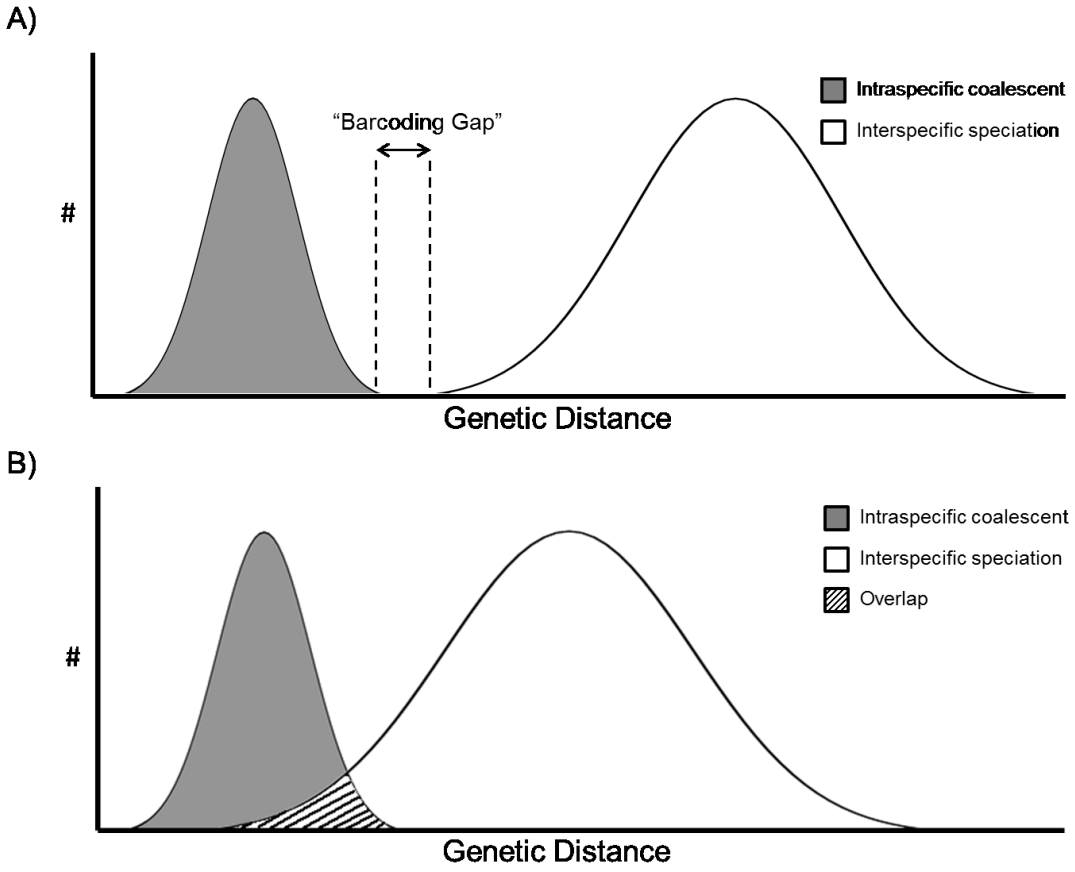

Figure 2.5: Chart showing an interaction between intraspecific coalescents and interspecific speciation. (A) indicates the presence of a DNA "Barcoding Gap" when there is no overlap between intra- and interspecific genetic variation; (B) depicts the absence of the "Barcoding Gap" when there is an overlap between intra- and interspecific genetic divergence. Figure adopted from Meyer and Paulay 2005.

2.4.3 Prospects of DNA barcoding on *Kappaphycus* and *Eucheuma*

The feasibility of DNA barcoding on seaweeds was proven to be promising in marine macroalgae, many of which involve cryptic species, or exhibit plasticity in terms of morphology throughout their diphasic or triphasic life cycles. Attracted by the clear-cut and straightforward mechanisms of DNA barcoding, phycologists have attempted to genetically barcode and archive DNA from a broad range of seaweeds, including the Gelidiales (Freshwater et al. 2010), Gigartinales (Clarkston and Saunders 2010; Le Gall and Saunders 2010; Saunders 2008), Graciliariales (Kim et al. 2010; Saunders 2009), Laminariales (McDevit and Saunders 2010), and Fucales (Kucera and Saunders 2008). Larger scale DNA barcoding efforts were also initiated with satisfactory results (Robba et al. 2006; Saunders 2005; Sherwood et al. 2010), offering valuable insights into the evolutionary ancestry of rhodophytes.

The establishment of a DNA barcode library for *Kappaphycus* and *Eucheuma* would prove most useful considering the drawbacks of morphology in differentiating members of the genera. These setbacks are generally caused by: (1) the phenotypic variability of *Kappaphycus* and *Eucheuma*, influenced largely by environmental conditions; (2) triphasic alternation of generations, where testrasporophytic and non-fertile gametophytic seaweeds are morphologically similar (Doty 1985, 1988; Doty and Norris 1985); (3) small thalli that lack morphological characters; and (4) shrinkage and loss of structure in herbaria. This study gauges the efficiency of various molecular markers as potential DNA barcodes for *Kappaphycus* and *Eucheuma*, and also investigates the applications that follow with the setting up of a DNA barcode library for these commercially important carrageenophytes.

CHAPTER 3: MATERIALS AND METHODS

3.1 Field sampling

Kappaphycus and *Eucheuma* samples were collected from various locations in Malaysia (Figure 3.1), where a majority were sampled from the islands around the East coast of Sabah (Semporna, Sabangkat, Omadal, Karindingan, Sisipan), and a few from the Pangkor islands of Peninsular Malaysia. Cultivated specimens (with their local names recorded) were collected directly from seaweed lines with consent from the respective farm owners, whereas wild specimens were obtained via snorkeling or scuba diving. Wild specimens in this context refer to specimens collected far from known cultivation sites. Non-local samples were also obtained from collaborators for DNA barcode assessment.

Seaweeds collected were labeled, tagged and kept in polyethylene plastic bags with proper aeration and humidity prior to processing upon docking. Fresh samples were photographed so as to digitally archive the "true" morphology as *Kappaphycus* and *Eucheuma* tend to shrink and become morphologically altered upon drying. Subsequently, the samples were washed with clean, artificial seawater to rid them of epiphytes, tiny marine organisms, and residual debris. For each sample, a small portion (~2-3 cm) of the tips were excised from the main thallus, blotted dry with SCOTT® C-Fold towels (Kimberly-Clark, USA), and kept in ziplock bags filled with silica gel; whereas the main thallus was pressed and air-dried. Silica gels and C-Fold towels were replaced at intervals to ensure dryness of the samples, to avoid fungal contamination.

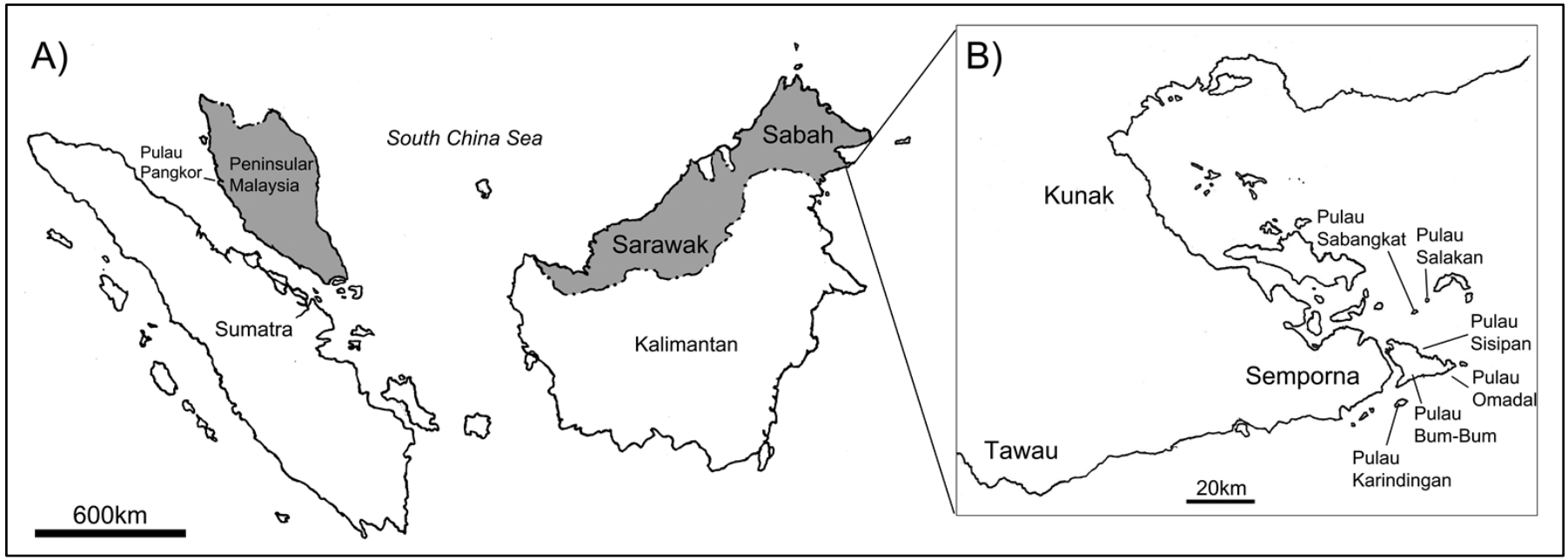

Figure 3.1: Locations of sampling sites in Malaysia. (A) shows the map of Peninsular Malaysia, Sabah and Sarawak, whereas (B) indicates an enlarged map of Southeastern Sabah, where most of the country's seaweed cultivation sites are concentrated.

3.2 Morphological observations

For comparison amongst various local varieties of *Kappaphycus* and *Eucheuma*, the gross morphology of samples collected were observed and recorded, with emphasis mainly on the overall plant size, thalli diameter, branching patterns and reproductive structures (if applicable). Microscopic observations were omitted considering the paucity of distinctive characters for differentiating between members of the genera (Doty 1988).

3.3 DNA extraction

The isolation of DNA from specimens was carried out using the commercially available i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, Korea). An approximate 1 cm of dried *Kappaphycus* or *Eucheuma* from the silica gel samples was used as starting material. With the use of liquid nitrogen, the starting material was carefully pulverized using a micropestle. This was followed by the addition of lysis buffer as well as RNAase and Proteinase K to degrade undesired RNA and proteins respectively. The resulting mixture was vortexed vigorously before being incubated at a temperature of 65 ˚C for an hour. Samples were vortexed at intervals of 5 minutes to enable proper agitation and mixing. Subsequently, a precipitation buffer PPT was added, mixed, and incubated on ice for 5 minutes to induce precipitation of solid matters within the solution. The hardened and glutinous mixture was then subjected to centrifugation (13,000 rpm) for 5 minutes and the supernatant mixed with a binding buffer prior to transfer into a spin column. The spin column was then centrifuged at 13,000 rpm for 1 minute, flow-through discarded, and then washed with washing buffers. The collection tube of the spin column was discarded, and the DNA eluted from the spin column's filter into a 1.5 ml tube using an elution buffer. The eluted sample was later kept at -20 ˚C for long term storage.

3.4 Polymerase Chain Reaction (PCR) amplification

PCR amplifications were performed for five molecular markers, namely the mitochondrial *cox*1, *cox*2, *cox*2-3 spacer and the plastid *rbc*L and RuBisCO spacer. The *cox*2-3 spacer and RuBisCO spacer were used specifically for phylogenetic analysis of Malaysian *Kappaphycus* and *Eucheuma*, whereas the *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L were used for marker assessments as potential DNA barcodes (Part B). Primer sets for each genetic marker are shown in Table 3.1.

An i-Taq™ Plus DNA Polymerase Kit (iNtRON Biotechnology, Korea) was used for PCR. The reaction mixtures for PCR are in volumes of 20 µl, as summarized in Table 3.2.

PCR was carried out using a Labnet MultiGene™ Gradient Thermal Cycler (Labnet, USA). The associated parameters utilized (Table 3.3) were either as suggested by the authors (refer to Table 3.1) or were slightly modified to achieve optimized conditions for the current set of equipment.

Table 3.1: Primer details and corresponding annealing temperatures for the *cox*1, *cox*2, *cox*2-3 spacer, *rbc*L and RuBisCO spacer molecular markers.

DNA Markers	Primers	Primer Sequences*	Annealing Temperatures, T_m	References
*cox*1	COXI43F	5'-TCAACAAATCATAAAGATATTGGWACT-3'	52 ˚C	Ger, Yan
	C622F	5'-CCTGTNTTAGCAGGWGCTATTACAATGC-3'		
	C880R	5'-ACAGTATACATATGATGNGCTCAAAC-3'		
	COXI1549R	5'-AGGCATTTCTTCAAANGTATGATA-3'		
*cox*2-3 spacer	*Cox*2_for	5'-GTACCWTCTTTDRGRRKDAAATGTGATGC-3'	50 ˚C	Zuc[1]
	*Cox*3_rev	5'-GGATCTACWAGATGRAAWGGATGTC-3'		
*cox*2	*Kcox*2_F71	5'-TTCAAGATCCTGCAACTCC-3'	51 ˚C	Present Study
	*Kcox*2_R671	5'-ATTTCACTGCATTGGCCAT-3'		
*rbc*L	F-7	5'-AACTCTGTAGTAGAACGNACAAG-3'	50 ˚C	Fre, Gav
	F-577	5'-GTATATGAAGGTCTAAAAGGTGG-3'		
	R-753	5'-GCTCTTTCATACATATCTTCC-3'		
	R-rbcS start	5'-GTTCTTTGTGTTAATCTCAC-3'		
RuBisCO	RBS1	5'-TGTGGACCTCTACAAACAGC-3'	52 ˚C	Mag, Zuc[2]
spacer	RBS2	5'-CCCCATAGTTCCCAAT-3'		

[1] Fre= Freshwater and Rueness (1994); Gav= Gavio and Fredericq (2002); Ger= Geraldino et al. (2006); Mag= (Maggs et al. 1992); Yan= Yang et al. (2007); Zuc[1]= Zuccarello et al. (1999; 2006); Zuc[2]= (Zuccarello et al. 1999b)

Table 3.2: Components of a 20µl PCR reaction

Ingredients	Amount
DNA	1ng- 50ng
10x PCR buffer	2µl
dNTP mixture (2.5mM each)	2µl
Primer 1	10pmoles
Primer 2	10pmoles
i-Taq™ DNA Polymerase (5U/µl)	0.2-0.5µl
Sterilized distilled water	Top up to 20µl

Table 3.3: PCR parameters for the *cox*1, *cox*2, *cox*2-3 spacer, *rbcL* and RuBisCO spacer DNA markers

	PCR Parameters														
DNA Markers	*cox*1			*cox*2			*cox*2-3 spacer			*rbc*L			RuBisCO spacer		
PCR Steps	Temp. (˚C)	Time (min)	Cycles	Temp. (˚C)	Time (min)	Cycles	Temp. (˚C)	Time (min)	Cycles	Temp. (˚C)	Time (min)	Cycles	Temp. (˚C)	Time (min)	Cycles
Pre-denaturation	94	4		94	4		94	4		94	4		94	4	
Denaturation	94	1		94	1		94	1		94	1		94	1	
Annealing	45	1	5	51	1	30	45	1	5	51	0.5	35	45	1	5
Elongation	72	1		72	1		72	1		72	1.5		72	1	
Post-elongation				72	10					72	10				
Denaturation	94	1					94	1					94	1	
Annealing	52	1	30				50	1	30				52	1	30
Elongation	72	1					72	1					72	1	
Post-elongation	72	10					72	10					72	10	

[1] Grey areas indicate non-applicable data

3.5 Gel electrophoresis

PCR amplicons were subjected to a 1% agarose gel electrophoresis to check for successful amplifications as well as to quantify the respective concentrations of amplified DNA. 50 g of agarose powder (1st Base, Malaysia) were mixed with 50 ml of Tris-Acetate-EDTA (TAE) buffer prior to boiling using a microwave oven (Sanyo, Japan). Upon cooling (~60 ˚C), 5 µl of SYBR®Safe DNA gel stain (Invitrogen, USA) was added and the resulting solution was cast onto the gel electrophoresis tray, comb inserted and left to cool down and solidify. 3 µl of samples were mixed with a 2 µl of 6x gel loading dye before being loaded into respective sample wells; a 1 kb ladder (Bioron, Germany) was used as reference to estimate the DNA length of amplified products. Gel electrophoresis was carried out using a Gel XL ENDURO set (Labnet, USA) at 100 volts for 10 minutes. Upon completion, the gel was taken out and viewed under UV light using an Alpha Imager 2200 gel documentation system (Alpha Innotech, USA). Samples were annotated using the system before being printed out as electrophoretograms using a Sony Thermal Printer (Sony, Japan).

3.6 DNA purification and DNA sequencing

DNA purification basically removes all undesired by-products of PCR apart from the amplicons. PCR products with specific amplifications i.e. only a single product (single band under UV) were purified using PCR purification; whereas samples with non-specific amplifications (more than one DNA product) were purified using gel purification. Despite the differences, both purification methods were carried out using a LaboPass™ (Cosmo Gentech, Korea) Gel and PCR Purification Kit, albeit with a minor change in protocols. PCR purification involved direct binding of PCR products to the provided spin columns, followed by washing and finally elution of DNA into 1.5ml tubes. Gel purification, on the other hand, first involved another gel electrophoresis of

the total volume of PCR product, and the desired band (identified based on reference to the 1kb ladder) was excised using a clean surgical blade under UV illumination, before continuing the mentioned steps for PCR purification. The purified PCR products were finally outsourced to Lucigen (Taiwan) for ABI-SOLiD (Sequencing by Oligonucleotide Ligation and Detection) DNA sequencing.

3.7 Data analysis

Sequencing results for all molecular markers were processed using Chromas Pro V1.5 (Technelysium Pty Ltd), where both the forward and reverse electropherograms for each sample were assembled to form contigs. Contigs were then checked for nucleotide ambiguities and corrected where applicable, and eventually saved as FASTA-format DNA sequences. In the meantime, all relevant DNA sequences available in GenBank for the *cox*1, *cox*2, *cox*2-3 spacer, *rbc*L and the RuBisCO spacer marker were downloaded. All DNA sequences of the same marker were compiled together as a .txt format file and appended into ClustalX V2.0 (Larkin et al. 2007) to generate multiple sequence alignments (MSA). Sequences with longer lengths were subsequently truncated, thus forming a MSA block which was then outputted as NEXUS format. Contents of the NEXUS file were later converted into sequential format using ALTER (ALignment Transformation EnviRonment) (Glez-Pena et al. 2010). The resulting file would be the basis of the subsequent analyses which are divided into two parts- (1) Molecular Taxonomy and Phylogenetics; and (2) DNA Barcode Assessment and DNA Barcoding.

3.7.1 Molecular taxonomy and phylogenetics

Maximum Parsimony (MP) trees were generated using PAUP* 4.0b10 (Swofford 2003) based on the heuristic search algorithm with the following parameters: 1,000 bootstrapping replications; with 100 stepwise random sequence addition and tree bisection reconnection (TBR) branch swapping. All characters were designated unordered and unweighted.

For Maximum Likelihood (ML) and Bayesian Inference (BI) analyses, the earlier NEXUS DNA sequence file was first annotated with suffixes "_P" for protein coding genetic markers prior to input into Kakusan v3 (Tanabe 2007), which generates best fit models (Akaike Information Criterion for ML, Bayesian Information Criterion for BI) for the mentioned analyses.

Best fit models as identified by Kakusan v3 for the Akaike Information Criterion are as follows: TN93 + Gamma model for the *cox*1 dataset; J1 + Gamma model for *cox*2; HKY85 + Gamma model for *cox*2-3 spacer; TIM + Gamma model for both *rbc*L and the RuBisCO spacer datasets. On the other hand, best fit models for the Bayesian Information Criterion are as follows: HKY85 + Gamma model for the *cox*1 dataset; HKY85 + Gamma model for *cox*2; HKY85 + Gamma model for *cox*2-3 spacer; GTR + Gamma model for *rbc*L and the HKY85 + Gamma model for the RuBisCO spacer dataset.

ML trees were generated using TreeFinder ver. Oct 2008 (Jobb et al. 2004) whereas BI trees were produced via Mr. Bayes 3.2.1 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). Using the Kakusan v3-generated model files, a phylogram was first generated using the Reconstruct Phylogeny module in TreeFinder. ML bootstrap supports were subsequently derived through 1,000 ML bootstrap replicates with 50% consensus level using the Bootstrap Analysis also available in TreeFinder. On the other

hand, the BI analyses were conducted with two sets of four Markov chain Monte Carlo (MCMC) chains, which were performed in parallel over a generation number of 2,000,000. Trees were sampled every 500th generation. Convergences of log likelihood values were tested using Tracer v1.5 (http:// 196 tree.bio.ed.ac.uk/software/tracer/) and burn-ins were discarded accordingly.

Neighbor-Joining (NJ) trees were also constructed for DNA barcode assessment. This was done using PAUP* 4.0b10 as well, where the Kimura 2-parameter (K2P) was used as the model for the generation of trees. Nodal supports were generated via 1,000 NJ bootstrap replicates.

All phylogenetic trees were viewed and processed using Figtree v1.3.1 (Rambaut A and Drummond A). For consistency, all trees were rooted with appropriate outgroups and arranged with decreasing node orders. Clade annotations for all phylogenetic trees were synchronized for easier referencing.

3.7.2 Haplotype analysis

Haplotype networks were constructed for the *cox*2-3 spacer genetic marker with the most GenBank entries. Gene genealogies at population level were computed using TCS 1.2.1 (Clement et al. 2000). Analyses were performed separately for *Kappaphycus* and *Eucheuma denticulatum*, inclusive of haplotypes and nomenclatures earlier reported (Halling et al. 2012; Zuccarello et al. 2006). Unrelated specimens, singletons as well as shorter length sequences within the dataset were omitted prior to analysis.

3.7.3 DNA barcode assessment and DNA barcoding

Considering the poor taxonomic understanding of *Kappaphycus* and *Eucheuma*, certain assessments on the proposed molecular markers were performed using two specific taxonomic groupings: (1) Operational Taxonomic Units (OTU) (Meyer 2004); and (2) non-OTU. The term OTU in this study represents a cluster of species-specific specimens constituting a monophyletic clade with (1) sufficient genetic variation from the sister taxa such that bifurcating branches are observed; or (2) distinct geographical assortment. Non-OTU is only used for the subsequent Large Dataset Assessment (Section 3.7.2.2), and basically involves the application of original and conventionally classified species names, most of which were derived based on morphological conformations to known type descriptions. Morphological characters were not used as criteria for OTU groupings due to the phenotypically plastic nature of *Kappaphycus* and *Eucheuma*, which will undoubtedly affect the accuracy of DNA barcoding.

3.7.3.1 Assessment of potential DNA barcodes

Potential DNA barcodes, namely the mitochondrial *cox*1, *cox*2, and *cox*2-3 spacer as well as the plastid *rbc*L genetic marker (the RuBisCO spacer was reported to be inferior in terms of resolving power by Zuccareollo and collaborators (2006) and was thus omitted) were tested and gauged through a series of assessments for their suitability as DNA barcodes for *Kappaphycus* and *Eucheuma*. Several samples of each *Kappaphycus* and *Eucheuma* species were selected for DNA barcode assessment, with priority given to samples of different localities. These include distance-based assessments and tree-based assessments. Both assessments require the aforementioned NEXUS format DNA dataset file for each molecular marker (with GenBank sequences excluded for consistency) as the starting file.

Distance-based analyses were carried out using TaxonDNA's Species Identifier v1.7.7 (Meier et al. 2006), where the pairwise distances for intraspecific and interspecific genetic distances were calculated using (1) the uncorrected pairwise distance; and (2) K2P corrected pairwise distances. The genetic distances, computed as (1) total overlap range; and (2) 90% overlap range with the exclusion of 5% of the largest intraspecific and 5% of the lowest interspecific samples, were then plotted to verify the existence of the "Barcoding Gap". Base pairs in common for each molecular marker were set to a minimum of 300 bp. The identification success of each molecular marker was tested using the *Best Match* (BM) and *Best Close Match* (BCM) criteria within the program. BM assigns a species name to the query based on its best barcode match, regardless of percentage similarity. The BCM criterion, being relatively stricter, compares the best barcode match to the query, and assigns a species name when a certain similarity threshold was attained (Meier et al. 2006). The smallest interspecific distance generated for each molecular marker dataset using the Pairwise Summary module was set as the threshold value for BM and BCM analyses. An additional All Species Barcode (ASB) distance analysis was also conducted specifically for the Large Data Assessment (Section 3.7.2.2) considering the larger dataset. Successful identifications for ASB involve query matches to at least two conspecific barcodes of the species tested. Queries that matched with allospecific barcodes were considered as misidentification, whereas queries followed by only one conspecific barcode or only a portion of the conspecific sequences were labeled as ambiguous (Meier et al. 2006). Genetic distances of each molecular marker were also computed using PAUP* 4.0b10 with default settings as additional reference.

Tree-based assessments were performed by the construction of NJ trees for each genetic marker as described under Section 3.7.1. Additional ML, MP and BI trees were also computed as supplementary data. Identification success was determined based on

guidelines and criteria described by Hebert et al. (2003a) and Meier et al. (2006). According to the former, successful identifications involve the query clustering along with all conspecific sequences within the phylogenetic tree; whereas failed identifications involve query-matched sequences occurring in multiple clusters or clades (non-monophyly). Queries that pair with sequences that occur in singularity or singletons, were designated ambiguous. Meier and co-workers (Meier et al. 2006) applied a relatively stricter tree-based identification criteria, where queries were labeled correctly identified when in polytomy with conspecifics, or at least one node into a clade of conspecifics. Queries in polytomy with only allospecific sequences, or those one node into an allospecific clade were considered misidentified. Queries were designated as ambiguous or unidentified when occurring without any conspecific sequences; or when occurring as a sister taxa to conspecifics.

3.7.3.2 Large dataset assessment

The Large Dataset Assessment empirically tests the efficiency of a molecular marker, preferably the most commonly used one i.e. *cox*2-3 spacer, when analyzed along with relatively larger amounts of samples. Specifically, this assessment tests the effect of the increased dataset on the inter- and intraspecific genetic divergences, and its effect on the "Barcoding Gap" which may lead to a change in the accuracy of species identification. To reveal the true potential of the genetic marker in terms of species identification, analyses were conducted with both OTU and non-OTU assortment of taxa. These taxa groupings were also tested using similar tree-based and distance-based DNA identification methods explained in Section 3.7.2.1, with an additional ASB test for the latter.

CHAPTER 4: RESULTS

4.1 Field sampling

Samples collected in this study are summarized in Table 4.1. Specimens were tentatively identified based on morphological descriptions from scientific literature; respective local names for each cultivated specimen were also obtained from local farmers and fisheries officers. A majority of farmed cultivars collected were easily recognized as *Kappaphycus*. Wild specimens, be it fertile or not, are relatively harder, if not impossible, to identify solely based on morphology, thus merely given tentative species names.

Non-local specimens were also obtained from respective collaborators from the Philippines, Indonesia and Vietnam.

Table 4.1: Details of samples used in this dissertation

No.	Sample Name	Operational Taxonomic Unit (OTU)	Sampling Location	Collection Code	GenBank Accession Numbers				
					*Cox*1	*Cox*2-3 spacer	*Cox*2	*rbc*L	RuBisCO spacer
1	*Kappaphycus alvarezii* 13 "*Buaya*" *	KA1	Sabangkat, Sabah, Malaysia	PSM11996-UMSS0144	-	JN663762	-	-	JN663731
2	*Kappaphycus alvarezii* 18 "*Tambalang Giant*" *		Sabangkat, Sabah, Malaysia	PSM12001-UMSS0154	-	JN663768	-	-	JN663737
3	*Kappaphycus alvarezii* 52 "*Buaya*" *		Omadal, Sabah, Malaysia	PSM12029-UMSS0196	-	JN663763	-	-	JN663732
4	*Kappaphycus alvarezii* 53 "*Tangan-tangan*" *		Omadal, Sabah, Malaysia	PSM12030-UMSS0198	-	JN663773	-	-	JN663742
5	*Kappaphycus alvarezii* 58 "*Tangan-tangan*" *		Omadal, Sabah, Malaysia	PSM12035-UMSS0203	JX624014	JN663774	JX624043	JX623985	JN663743
6	*Kappaphycus alvarezii* 63 "*Tambalang Giant*"		Sisipan, Sabah, Malaysia	PSM12043-UMSS0214		JN663769		-	JN663638
7	*Kappaphycus alvarezii* 89 "*Tambalang* Brown" *		Sandakan, Sabah, Malaysia	PSM12059-UMSS0230	JX624015	JN663766	JX624044	JX623986	JN663735
8	*Kappaphycus alvarezii* 103*		Sabangkat, Sabah, Malaysia	PSM12072-UMSS0243	JX624016	JN663776	JX624045	JX623987	JN663745
9	*Kappaphycus alvarezii* 109 "*Tangan-tangan*"		Semporna, Sabah, Malaysia	PSM12078-UMSS0249	-	JN663775	-	-	JN663744
10	*Kappaphycus alvarezii* 121 "*Tambalang* Green" *		Pangkor Island, Perak, Malaysia	PSM12105-UMSS0260	JX624017	JN663772	JX624046	JX623988	JN663741
11	*Kappaphycus alvarezii* 123 "*Tambalang* Brown" *		Pangkor Island, Perak, Malaysia	PSM12107-UMSS0262	-	JN663767	-	-	JN663736
12	*Kappaphycus alvarezii* BA*		Semporna, Sabah, Malaysia	-	-	JN234760	-	-	-
13	*Kappaphycus alvarezii* BN*		Semporna, Sabah, Malaysia	-	-	JN234759	-	-	-
14	*Kappaphycus alvarezii* YF*		Semporna, Sabah, Malaysia	-	-	JN234762	-	-	-
15	*Kappaphycus alvarezii* 433		Teluk Ekas, Indonesia	PSM12290-UMSS0433	JX624018	JX624072	JX624047	JX623989	-
16	*Kappaphycus alvarezii* ZAM4 "*Milo*"		Zamboanga City, Mindanao, Philippines	AQHZAM004-UMSS0380	JX624019	JX624073	JX624048	JX623990	-
17	*Kappaphycus alvarezii* V7 "Dark Green"		Son Hai, Vietnam	PSM12380-UMSS0525	JX624020	JX624074	JX624049	JX623991	-
18	*Kappaphycus alvarezii* E3*		Venezuela	-	-	AY687427	-	-	AY687410
19	*Kappaphycus alvarezii* 2614*		Hawaii	-	FJ554853	FJ554862	-	-	-
20	*Kappaphycus alvarezii* UR13*		Tanzania	-	-	JQ713902	-	-	-
21	*Kappaphycus alvarezii* 1999C		Philippines	-	-	-	-	AF489870	-
22	*Kappaphycus alvarezii* 1999N		Philippines	-	-	-	-	AF489872	-
23	*Kappaphycus alvarezii*		Philippines	-	-	-	-	AF099694	-
24	*Kappaphycus alvarezii* E130*	KA2	Tanzania	-	-	AY687427	-	-	-
25	*Kappaphycus alvarezii* Reef4*		Paje-Jambiani, Tanzania	-	-	JQ713901	-	-	-
26	*Kappaphycus alvarezii* E16*		Madagascar	-	-	AY687430	-	-	AY687415
27	*Kappaphycus alvarezii* E57*	KA3	Hawaii	-	-	AY687432	-	-	AY687413
28	*Kappaphycus alvarezii* E71*		Hawaii	-	-	AY687433	-	-	-
29	*Kappaphycus alvarezii* 919*		Hawaii	-	-	FJ554860	-	-	-
30	*Kappaphycus alvarezii "Bola-bola"*		Philippines	-	EU334416	-	-	-	-
31	*Kappaphycus "alvarezii"* 3954		Hawaii		FJ554856	-	-	-	-

Table 4.1, continued

No.	Sample Name	Operational Taxonomic Unit (OTU)	Sampling Location	Collection Code	GenBank Accession Numbers				
					*Cox*1	*Cox*2-3 spacer	*Cox*2	*rbc*L	RuBisCO spacer
32	*Kappaphycus "alvarezii"* 3955*	KA3	Hawaii	-	FJ554857	FJ554861	-	-	-
33	*Kappaphycus "alvarezii"* 3956		Hawaii	-	FJ554858	-	-	-	-
34	*Kappaphycus "alvarezii"* 3957		Hawaii	-	FJ554854	-	-	-	-
35	*Kappaphycus* sp. "*Bola-bola*"	-	Philippines	-	-	-	-	-	EU334427
36	*Kappaphycus striatus* 1 "Yellow Flower" *	KS1	Sabangkat, Sabah, Malaysia	PSM11984-UMSS0128	JX624021	JN663779	JX624050	JX623992	JN663748
37	*Kappaphycus striatus* 31 "Green Flower" *		Sabangkat, Sabah, Malaysia	PSM12011-UMSS0170	JX624022	JN663780	JX624051	JX623993	JN663749
38	*Kappaphycus striatus* 59 "Green Flower" *		Bum-Bum Island, Malaysia	PSM12039-UMSS0208	JX624023	JN663777	JX624052	JX623994	JN663746
39	*Kappaphycus striatus* 60 "Green Flower" *		Bum-Bum Island, Malaysia	PSM12040-UMSS0209	-	JN663778	-	-	JN663747
40	*Kappaphycus striatus* AG*		Semporna, Sabah, Malaysia	-	-	JN234763	-	-	-
41	*Kappaphycus striatus* GF*		Semporna, Sabah, Malaysia	-	-	JN234765	-	-	-
42	*Kappaphycus striatus* GTF*		Semporna, Sabah, Malaysia	-	-	JN234764	-	-	-
43	*Kappaphycus striatus* 460		Kertasari, Indonesia	PSM12293-UMSS0460	JX624024	JX624075	JX624053	JX623995	-
44	*Kappaphycus striatus* GUI4 "*Cottonii*"		Guimaras Is. Panay, Philippines	AQHGUI004-UMSS0360	JX624025	JX624076	JX624054	JX623996	-
45	*Kappaphycus striatus* SIT5 "*Cottonii* light green (sacol)"		Sitangkai, Tawi Mindanao, Philippines	AQHSIT005-UMSS0394	JX624026	JX624077	JX624055	JX623997	-
46	*Kappaphycus striatus* V6 "*Payaka* Green"		Cam Ranh, Vietnam	PSM12379-UMSS0524	JX624027	JX624078	JX624056	JX623998	-
47	*Kappaphycus striatus* E89*		Philippines	-	-	AY687434	-	-	AY687416
48	*Kappaphycus* sp. "*Tambalang*" HRM56gz		Philippines	-	EU334415	-	-	-	-
49	*Kappaphycus "Cottonii"*		Philippines	-	-	-	-	AF099695	-
50	*Kappaphycus "Cottonii"*		Philippines	-	-	-	-	AF481499	-
51	*Kappaphycus* sp. *"Sacol"*		Philippines	-	-	-	-	AF481500	-
52	*Kappaphycus "alvarezii"* 1999H		Philippines	-	-	-	-	AF489871	-
53	*Kappaphycus striatus* 83*	KS2	Sabangkat, Sabah, Malaysia	PSM12053-UMSS0224	JX624028	JN663781	JX624057	JX623999	JN663750
54	*Kappaphycus striatus* 98*		Sabangkat, Sabah, Malaysia	PSM12067-UMSS0238	JX624029	JN663782	JX624058	JX624000	JN663751
55	*Kappaphycus striatus* 105*		Sabangkat, Sabah, Malaysia	PSM12074-UMSS0245	JX624030	JN663783	JX624059	JX624001	JN663752
56	*Kappaphycus striatus* D13*		Semporna, Sabah, Malaysia	-	-	JN645177	-	-	-
57	*Kappaphycus striatus* D14*		Semporna, Sabah, Malaysia	-	-	JN645178	-	-	-
58	*Kappaphycus striatus* SIT4 "*Kab-kab* green"		Sitangkai, Tawi Mindanao, Philippines	AQHSIT004-UMSS0393	JX624031	JX624079	JX624060	JX624002	-
59	*Kappaphycus striatus* E117*		Indonesia	-	-	AY687435	-	-	-
60	*Kappaphycus striatus* E48*		Indonesia	-	-	AY687431	-	-	-
61	*Kappaphycus "alvarezii"* 2002H		Philippines	-	-	-	-	-	AF489868

Table 4.1, continued

No.	Sample Name	Operational Taxonomic Unit (OTU)	Sampling Location	Collection Code	GenBank Accession Numbers				
					*Cox*1	*Cox*2-3 spacer	*Cox*2	*rbc*L	RuBisCO spacer
62	*Kappaphycus* sp. 14 "*Aring-aring*" *	KAr	Sabangkat, Sabah, Malaysia	PSM11997-UMSS0146	-	JN663784	-	-	JN663753
63	*Kappaphycus* sp. 49 "*Aring-aring*" *		Sabangkat, Sabah, Malaysia	PSM12026-UMSS0192	JX624032	JN663785	JX624061	JX624003	JN663754
64	*Kappaphycus* sp. 93 "*Aring-aring*" *		Sabangkat, Sabah, Malaysia	PSM12063-UMSS0234	JX624033	JN663786	JX624062	JX624004	JN663755
65	*Kappaphycus* sp. 115 "*Aring-aring*"		Sabangkat, Sabah, Malaysia	PSM12100-UMSS0255	JX624034	JX624080	JX624063	JX624005	-
66	*Kappaphycus cottonii* E108		Philippines	-	-	AY687426	-	-	AY687409
67	*Kappaphycus cottonii* AOL186gz		Philippines	-	EU334417	-	-	-	-
68	*Eucheuma denticulatum* 44 "*Spinosum*" *	ED1	Sabangkat, Sabah, Malaysia	PSM12021-UMSS0187	JX624035	JN663787	JX624064	JX624006	JN663756
69	*Eucheuma denticulatum* 45 "*Spinosum*" *		Sabangkat, Sabah, Malaysia	PSM12022-UMSS0188	JX624036	JN663788	JX624065	JX624007	JN663757
70	*Eucheuma denticulatum* 46 "*Spinosum*" *		Sabangkat, Sabah, Malaysia	PSM12023-UMSS0189	-	JN663789	-	-	JN663758
71	*Eucheuma denticulatum* 56 "*Spinosum*" *		Omadal, Sabah, Malaysia	PSM12033-UMSS0201	JX624037	JN663790	JX624066	JX624008	JN663759
72	*Eucheuma denticulatum* 57 "*Spinosum*" *		Omadal, Sabah, Malaysia	PSM12034-UMSS0202	-	JN663791	-	-	JN663760
73	*Eucheuma denticulatum* 99 "*Spinosum*" *		Sabangkat, Sabah, Malaysia	PSM12068-UMSS0239	-	JN663792	-	-	JN663761
74	*Eucheuma denticulatum* DM*		Semporna, Sabah, Malaysia	-	-	JN234756	-	-	-
75	*Eucheuma denticulatum* AD*		Semporna, Sabah, Malaysia	-	-	JN980403	-	-	-
76	*Eucheuma denticulatum* AB*		Semporna, Sabah, Malaysia	-	-	JN234758	-	-	-
77	*Eucheuma denticulatum* E13*		Indonesia	-	-	AY687429	-	-	-
78	*Eucheuma denticulatum "Spaghetti"* HRM15gz		Philippines	-	EU334420	-	-	-	-
79	*Eucheuma denticulatum "Spaghetti"* HRM21gz		Philippines	-	EU334419	-	-	-	-
80	*Eucheuma denticulatum* 454	ED2	Kertasari, Indonesia	PSM12292-UMSS0454	JX624038	JX624081	JX624067	JX624009	-
81	*Eucheuma denticulatum* BOH5 "*Spinosum*"		Bohol, Central Visayas, Philippines	AQHBOH005-UMSS0371	JX624039	JX624082	JX624068	JX624010	-
82	*Eucheuma denticulatum* 41 "*Cacing"*		Sabangkat, Sabah, Malaysia	PSM12018-UMSS0181	JX624040	JX624083	JX624069	JX624011	Appendix E
83	*Eucheuma denticulatum* 42 "*Cacing"*		Sabangkat, Sabah, Malaysia	PSM12019-UMSS0183	JX624041	JX624084	JX624070	JX624012	Appendix F
84	*Eucheuma denticulatum* 97 "*Cacing"*		Sabangkat, Sabah, Malaysia	PSM12066-UMSS0237	JX624042	JX624085	JX624071	JX624013	Appendix G
85	*Eucheuma denticulatum* CG *		Semporna, Sabah, Malaysia	-	-	JN234757	-	-	-
86	*Eucheuma denticulatum* E32*		Indonesia	-	-	AY687437	-	-	-

Table 4.1, continued

No.	Sample Name	Operational Taxonomic Unit (OTU)	Sampling Location	Collection Code	GenBank Accession Numbers				
					*Cox*1	*Cox*2-3 spacer	*Cox*2	*rbc*L	RuBisCO spacer
87	*Eucheuma denticulatum* 888*		Hawaii	-	-	FJ554859	-	-	-
88	*Eucheuma denticulatum "endong"* AOL053gz		Philippines	-	EU334418	-	-	-	-
89	*Eucheuma denticulatum* 3953		Hawaii	-	FJ554855	FJ561733	-	-	-
90	*Eucheuma denticulatum* E8*	ED3	Madagascar	-	-	AY687428	-	-	-
91	*Eucheuma denticulatum* PAC5*		Paje-Jambiani	-	-	JQ713903	-	-	-
92	*Eucheuma denticulatum* E60*		Mauritius	-	-	AY687439	-	-	AY687414
93	*Eucheuma isiforme* E35	-	USA	-	-	AY687420	-	-	AY687403
94	*Eucheuma isiforme* E2	-	-	-	-	AY687421	-	-	AY687404
95	*Eucheuma isiforme* E37	-	USA	-	-	AY687419	-	-	AY687402
96	*Eucheuma isiforme*	-	USA	-	-	-	-	AF099691	-
97	*Eucheuma serra*	-	Taiwan	-	-	-	-	AF099692	-
98	*Eucheuma unicatum*	-	USA	-	-	-	-	AF099693	-
99	*Eucheuma platycladum* E65*	EP	Tanzania	-	-	AY687423	-	-	AY687406
100	*Eucheuma platycladum* E111*		Kenya	-	-	AY687422	-	-	AY687405
101	*Eucheuma* sp. E59	-	Hawaii	-	-	-	AY687425	-	AY687408
102	*Eucheuma* sp. E66	-	Kenya	-	-	-	AY687418	-	AY687401
103	*Eucheuma* sp. E110	-	Tanzania	-	-	-	AY687424	-	AY687407
104	*Betaphycus philippinensis* E118	-	Philippines	-	-	AY687417	-	-	AY687400
105	*Betaphycus philippinensis*	-	Philippines	-	-	-	-	AF099692	-
106	*Betaphycus speciosum*	-	Australia	-	-	-	-	AF099685	-
107	*Solieria* 120	-	Merambong, Johor, Malaysia	PSM12104-UMSS0259	-	JN663793	Appendix A	Appendix C	-
108	*Gracilaria changii* 98U	-	Sabangkat, Sabah, Malaysia	PSM122776-UMSS0696	-	-	Appendix B	Appendix D	-
109	*Gracilaria parvispora* G425	-	Korea	-	EF434921	-	-	-	-
110	*Hypnea pannosa* H0923	-	Australia	-	EU240820	-	-	-	-

[1] Specimens were grouped into Operational Taxonomic Units (OTU) for selected analyses.
[2] Dashes (-) indicate non-available or irrelevant data.
[3] Asterisks (*) indicate samples where corresponding *cox*2-3 spacer sequences were obtained from the GenBank and used for *Large Dataset Assessment*.

4.2 Morphological observations

Upon collection of all specimens, morphological characters used to differentiate between Malaysian *Kappaphycus* and *Eucheuma* varieties were described and shown in Table 4.2. Associated photos were illustrated as Figure 4.1-4.3.

Table 4.2: Morphological descriptions of local varieties of *Kappaphycus* and *Eucheuma* in Malaysia.

Sample	Variety	Nature	Color	Plant size	Branching Patterns	Texture
K. alvarezii	*Buaya (Crocodile)*	Domesticated	Brown, pale brown	<1 m	Thalli cylindrical (diameter <1.5 cm), branching irregular, indeterminate and mainly primary. Secondary branches small (diameter <0.5cm) and somewhat regularly spaced. Tertiary and quaternary branching rare and irregular. Branch apex often pointed or dichotomous.	Thalli robust and hard. Surface rough and cartilaginous. Characteristically dense, uneven and blunt protrusions apparent on main axis and primary branches- resembling crocodile scales.
	Tambalang Brown		Brown	<60 cm	Thalli cylindrical (diameter <1 cm), branching generally open, indeterminate, irregular to sympodial. Branching degree up to quaternary although scarce. Primary branching more frequent than *Buaya* variants. Secondary branches (diameter <0.5 cm) often regularly spaced. Branch length decreases with each level of branching. Terminal branches dichotomous, pointed to rounded.	Thalli fleshy. Surface smooth and cartilaginous.
	Tambalang Green		Green			
	Tambalang Giant		Brown	<1.5 m	Somewhat similar to *Tambalang Green* and *Tambalang Brown* in terms of morphology, apart from the larger size. Thalli cylindrical (diameter <1.5 cm), branching indeterminate and irregular. Primary branches dominant. Secondary branches dense, unilateral to irregular. Tertiary branches short and small (<0.5 cm); quaternary branches rare. Branch apices mostly slender and pointed.	Thalli robust and hard. Surface smooth and cartilaginous.
	Tangan-tangan		Brown, pale brown	<50 cm	Thalli cylindrical (diameter <0.9 cm). Branching open, irregular to sympodial. Primary and secondary branches often regularly spaced. Secondary and tertiary branches bifurcate or multifurcate into characteristically hand-like, outward radiating clusters of small, short and dense terminal branches. The "Candelabra" effect (Neish IC 2008) especially evident when exposed to unidirectional sunlight.	Thalli fleshy. Surface smooth and cartilaginous.
	Isolate 103	Wild	Yellowish brown	Approx. 25 cm	Holdfast and stipe not observed. Main axis, primary and secondary branches indeterminate, cylindrical and thick (diameter <1.3cm). Tiny, dense lateral branches (diameter <0.7 cm) irregular, pinnate to unilateral; abundant throughout thalli surface. Terminal branches often multifurcating and short.	Thalli thick and hard. Surface cartilaginous, rough and uneven. Denticulations present due to damaged tissue.

Table 4.2, continued

Sample	Variety	Nature	Color	Plant size	Branching Patterns	Texture
K. striatus	*Green Flower*	Domesticated	Green	<40 cm	Thallus roughly isodiametric and dorsally symmetrical. Thalli cylindrical. Basal stem (diameter <1.3 cm) often give rise to multiple primary branches that radiate outwards. Main axis not conspicuous. Branching angular, frequent and seldom more than 2cm apart with up to five degrees of branching. Secondary branches often irregular, subsequent degrees of branchings almost always bifurcating or trifurcating. Apical tips short and small (diameter <0.4cm), generally blunt- ended or rounded.	Thalli dense and compact, giving a cauliflower-like appearance. Surface smooth and cartilaginous, with occasional blunt protrusions and denticulations.
	Yellow Flower		Yellowish brown			
	Isolate 31	Wild	Green	Approx. 17 cm	Holdfast and stipe not observed. Main axis (diameter <0.8 cm) somewhat sympodial. Primary branches (diameter <0.6 cm) irregular and long. Secondary branches frequent, dense (<0.3 cm) and more or less regularly spaced. Subsequent branching dichotomous or irregularly dichotomous. Branch apex slender and pointy.	Thall fleshy, dense but pliable. Surface smooth and cartilaginous. Denticulations present due to damaged tissue.
	Isolate 83	Wild (cystocarpic)	Pale brown	Approx. 15 cm	Discoid holdfast (diameter ≈1 cm), stipe not apparent. Main axis sympodial. Primary (diameter <0.8 cm) and secondary (diameter <0.6 cm) branches irregular. Tertiary branches mostly short and blunt- ended. Terminal branches rounded.	Thalli robust and hard. Surface rough with fertile cystocarps. Cystocarps somewhat cone-shaped with pointy or rounded ends (diameter <0.2 cm), clustering throughout a majority of the thalli surface, whilst entirely absent in certain portions. Cystocarps may be present on branch apex.
	Isolate 98	Wild (cystocarpic)	Yellowish green	Approx. 23 cm	Holdfast and stipe not observed. Main axis (diameter <1 cm) sympodial. Primary and secondary branches dominant. Branching indeterminate and irregular, but somewhat regularly spaced. Terminal branches often blunt- ended or dichotomous.	Thalli fleshy and firm. Surface unevenly rough. Cystocarps somewhat cone-shaped with pointy or rounded terminals (diameter <0.2 cm), crowded in certain parts of the thalli. Cystocarps may be present on branch apex.
	Isolate 105	Wild (cystocarpic)	Yellowish brown	Approx. 19 cm	Discold holdfast (diameter ≈0.9 cm) with no apparent stipe; multiaxial. Primary (diameter <1 cm) and secondary (diameter <0.9 cm) irregular. Secondary, non-cystocarpic branches indeterminate, long and slender (diameter <0.4 cm); often dichotomous.	Thalli fleshy. Surface rough and uneven. Non-cystocarp bearing thalli smooth and soft. Cystocarps blunt to pointy (diameter <0.2 cm), abundant throughout axes and fertile branches. Cystocarps may be present on branch apex.

Table 4.2, continued

Sample	Variety	Nature	Color	Plant size	Branching Patterns	Texture
Kappaphycus sp.	*Aring-aring*	Domesticated	Yellowish brown, green	<50 cm	Main axes (diameter <1 cm) exhibit irregular furcation. Branches generally slimmer than those of *K. alvarezii* and *K. striatus*. Branching open, indeterminate and never directly opposite. Primary branches (diameter <0.6 cm) irregular, giving rise to smaller, irregularly arranged secondary branches (diameter <0.5cm). Tertiary branches (diameter <0.3cm) somewhat tapered with pointed apices which grow in a generally unilateral fashion towards light. Quaternary branching rare.	Thalli smooth, fleshy, cartilaginous and pliable.
	Isolate 93	Wild (cystocarpic)	Yellowish brown	Approx. 18 cm	Discoid holdfast. Multiaxial, only one axis (diameter ≈1.2cm) dominant and larger; new axes grow from or close to the main axis below the basal primary branch. Stipes (diameter <0.4 cm) gradually thicker from the holdfast. Primary (diameter <0.9 cm) and secondary (diameter <0.6 cm) branches irregular. Tertiary branching rare. Branch apex blunt-end or rounded.	Thalli cartilaginous and firm. Surface unevenly rough with blunt protrusions. Cystocarps somewhat hemispherical and irregularly scattered throughout the entire thalli. Matured cystocarps swollen and larger (diameter ≈0.2 cm); dark patches (carposporangium) apparent at the center of each hemisphere. Cystocarps may be present on branch apex.

Table 4.2, continued

Sample	Variety	Nature	Color	Plant size	Branching Patterns	Texture
E. denticulatum	*Spinosum*	Domesticated	Dark brown	<25 cm	Thalli cylindrical (diameter <0.8 cm) and generally smaller than *Kappaphycus*. Main axis apparent. Branches irregular, arising from occasional indeterminate spines and are often regularly spaced. Branching up to quaternary. Terminal branches slim, dichotomous or pointed.	Thalli brittle and crisp. Surface unequally rough, with characteristic pinnate or pectinate, simples spines (diameter <0.2 cm) throughout the thalli, particularly denser and orderly arranged on the rachis and primary branches. Spines often occur in whorls, where opposing spines are mostly determinate and generally similar in length. Spine frequencies and length decrease with each branching, terminal branches often axiferous.
	Cacing	Domesticated, Wild	Yellowish brown, brown	<25 cm	Thalli cylindrical (diameter <1 cm). Main sympodial. Branching irregular but more or less regularly spaced. Branching degree up to quaternary. Terminal branches rounded, with tiny, pointed, often bifurcating or multifurcating growth protrusions on the apex.	Thalli fleshy and cartilaginous. Surface smooth and slippery when wet. Spines simple, regularly spaced and often pinnate. Frequency of spines significantly lesser than that of *Spinosum* varieties but are evenly distributed and often widely spaced throughout the thalli surface. Spines mostly indeterminate.
	Isolate 56	Wild	Dark brown	Approx. 16 cm	Holdfast and stipe not observed. Thalli cylindrical; rachis (diameter <0.6 cm) apparent. Primary branches (diameter ≈0.4 cm) pinnate and dense. Secondary branches (diameter <0.2 cm) often pinnate to pectinate or unilateral. Branching often to tertiary level and may be axiferous. Branch apices slender and tapered.	Thalli brittle and crisp. Surface generally rough and uneven. Spineless thalli surface smooth and cartilaginous. Spines (diameter <0.15 cm) pinnate and dense, largely dominant on main axis and primary branches.
	Isolate 57	Wild	Reddish brown	Approx. 25 cm	Holdfast, stipe and main axis not conspicuous. Thalli cylindrical. Branching irregular but regularly spaced. Branches mainly primary (diameter <0.6 cm) and secondary (diameter <0.5 cm). Tertiary branches uncommon (diameter <0.3 cm).Branch apices mostly slender and pointed.	Thalli damaged, self-adhered and clumped. Thalli brittle and crisp. Surface unevenly rough. Spines (diameter <0.15 cm) circumferentially pinnate and generally determinate, abundant throughout thalli surface.
	Isolate 97	Wild	Yellowish green	Approx. 15 cm	Holdfast and stipe not observed. Thalli cylindrical. Main axis conspicuous and long. Primary (diameter <0.6 cm) and secondary (diameter <0.4 cm) branches pinnate to irregular and somewhat orderly spaced. Tertiary branches (diameter <0.3 cm) uncommon and short. Terminal branches slender and pointed.	Thalli brittle and crisp. Surface rough and uneven. Spines (diameter <1.5 cm) abundant, dense and circumferentially pinnate throughout primary and secondary branches. Spines less frequent and strictly pinnate on tertiary branches.

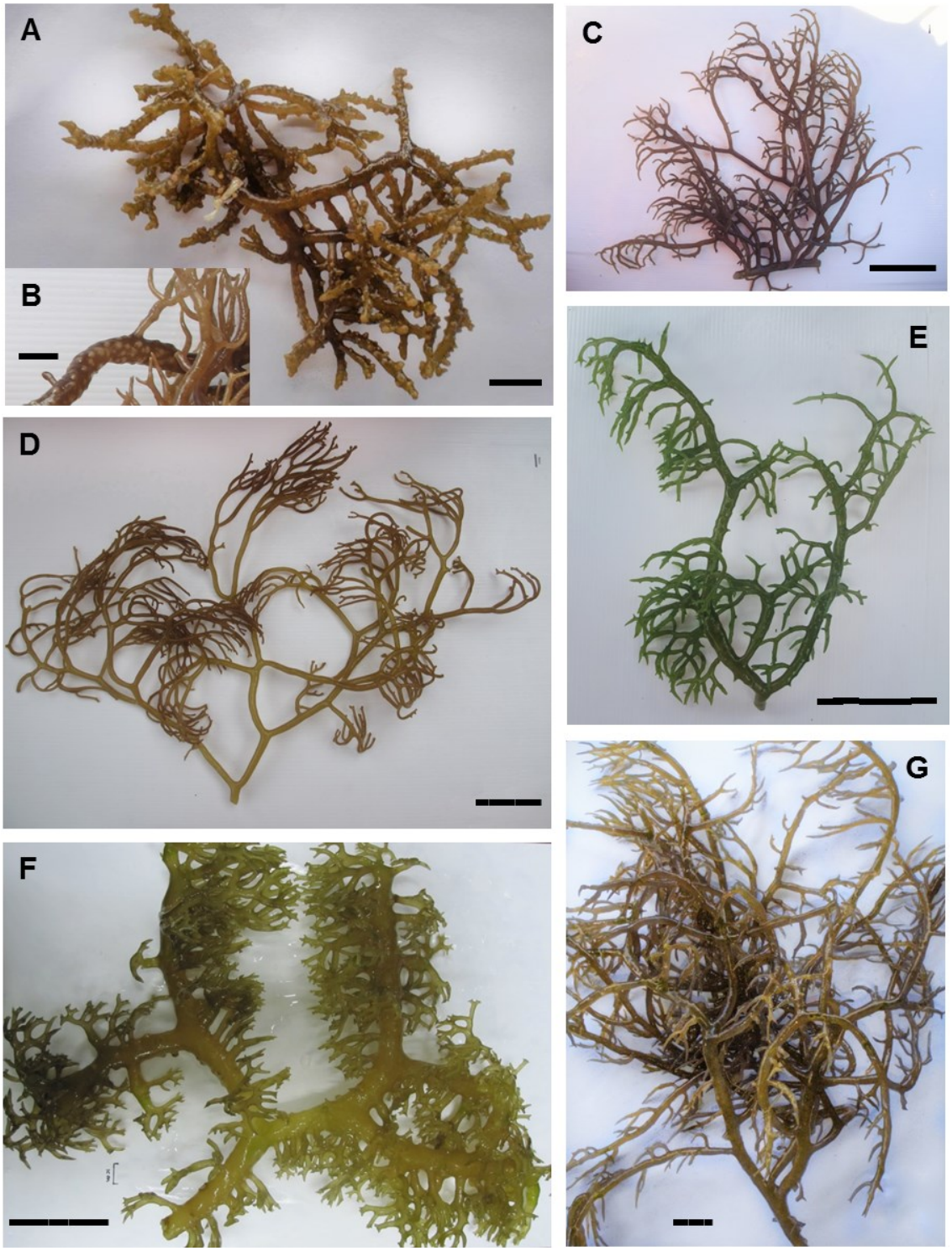

Figure 4.1: Local varieties of *Kappaphycus alvarezii* in Malaysia. **A**=*Tambalang Buaya* (Crocodile); **B**=Characteristic protrusions on *Buaya* varieties; **C**=*Tambalang Brown*; **D**=*Tangan-tangan*; **E**=*Tambalang Green*; **F**=Wild *K. alvarezii*, Isolate 103; **G**=*Tambalang Giant.* [b: scale bar=1 cm; a, c, d, e, f, g: scale bar=5 cm]

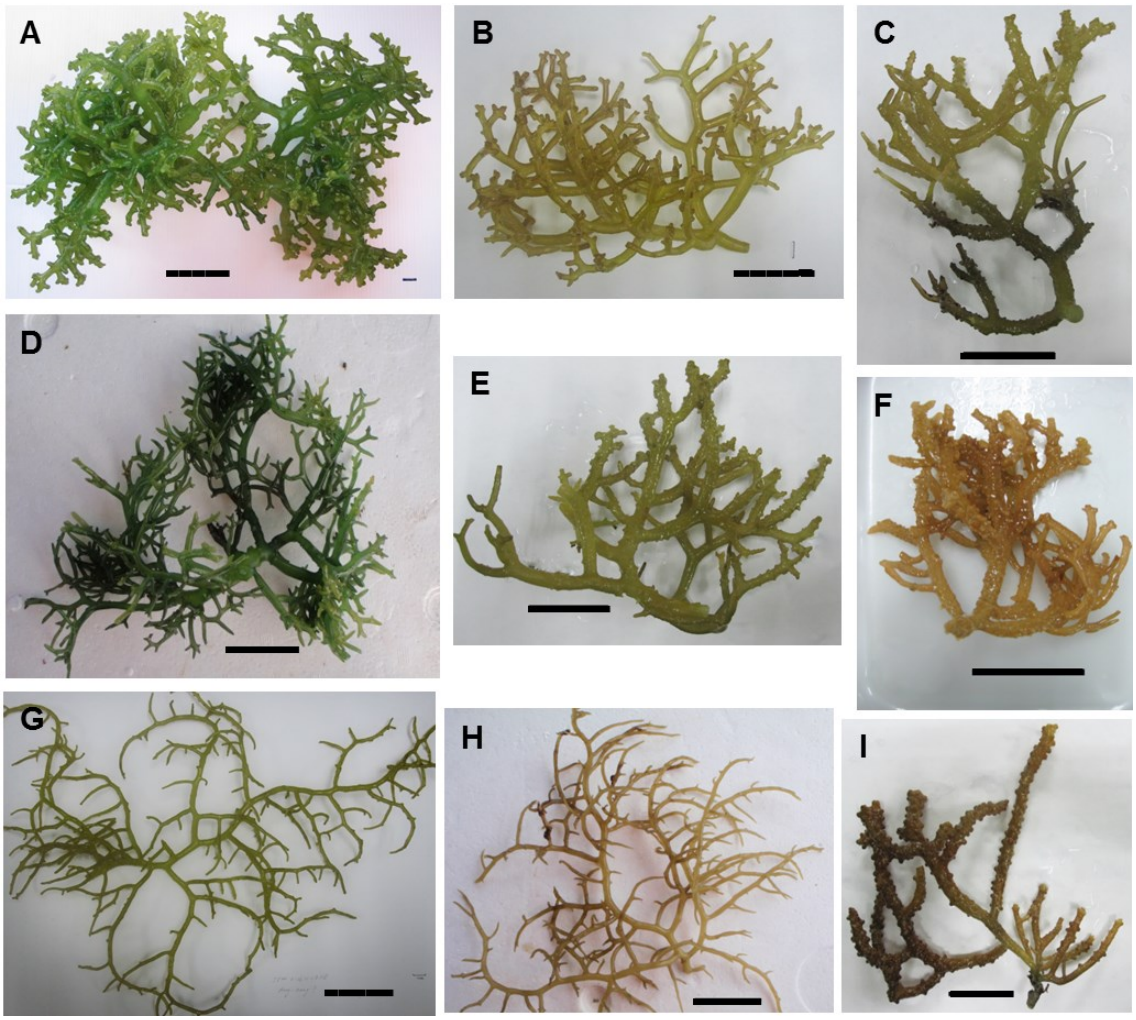

Figure 4.2: Local varieties of *Kappaphycus* in Malaysia. **A**=*Green Flower*; **B**=*Brown/Yellow Flower*; **C**=Wild, cystocarpic *K. striatus*, isolate 105; **D**=Wild *K. striatus*, isolate 31; **E**=Wild, cystocarpic *K. striatus*, isolate 98; **F**=Wild, cystocarpic *K. striatus*, isolate 83; **G**= *Aring-aring*, green; **H**=*Aring-aring*, brown; **I**=Wild, cystocarpic *Aring-aring*, isolate 93. [scale bar=5 cm]

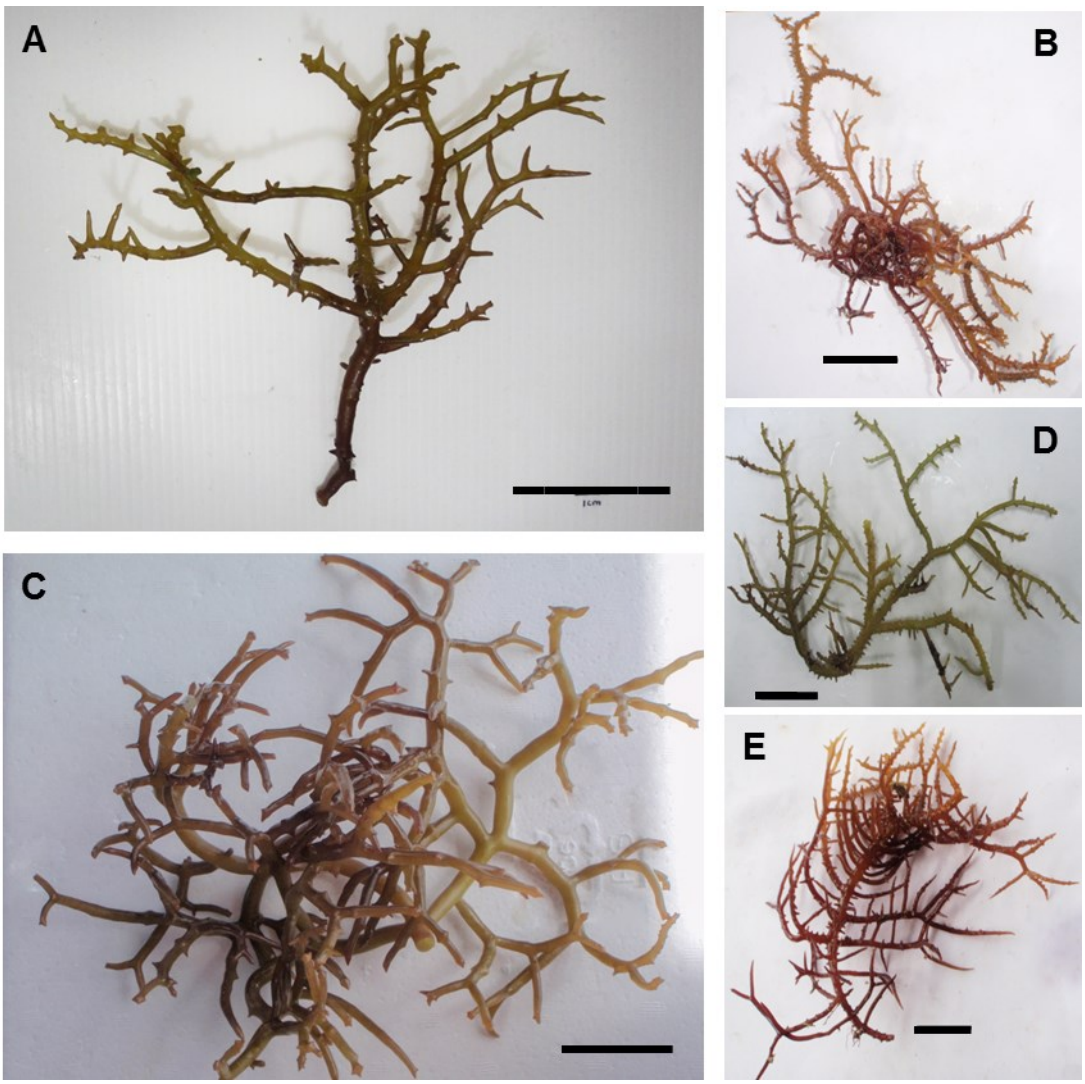

Figure 4.3: Local varieties of *Eucheuma* in Malaysia. **A**=*Spinosum*; **B**=Wild *E. denticulatum,* isolate 56; **C**=*Cacing*; **D**=Wild *E. denticulatum,* isolate 99; **E**=Wild *E. denticulatum,* isolate 57 [scale bar=5 cm].

4.3 DNA amplification and purification

DNA amplification was generally straightforward for most molecular markers, with most samples successfully producing amplicons over one PCR run. As for the *cox*1 and *rbc*L markers, nested primers were used on the PCR products from the first PCR run in order to obtain two separate but shorter desired DNA products, which overlaps at a particular region and can thus be combined to form a contig representing the desired full length of the genetic marker.

Almost all genetic markers produce single desired products over one PCR run. Electrophoretograms of each molecular marker and the respective amplicon sizes were shown in Figure 4.4 and 4.5. Non-specific amplifications were mostly observed only for nested *cox*1 and *rbc*L reactions and hence required gel purification.

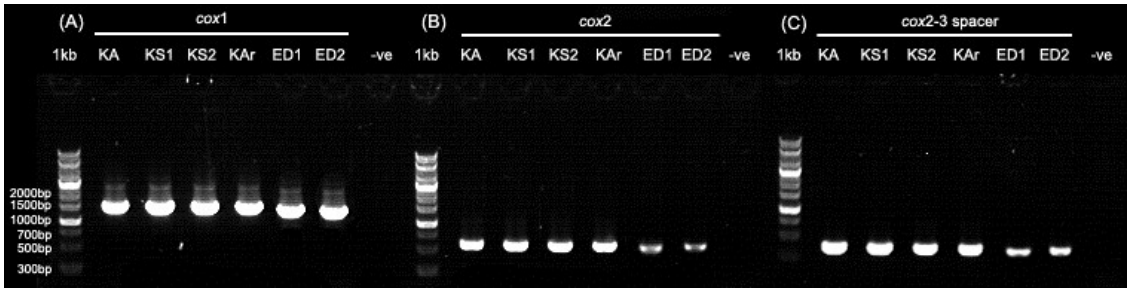

Figure 4.4: Electrophoretogram showing amplicons of the mitochondrial (A) *cox*1 [~1,400bp], (B) *cox*2 [~500bp] and (3) *cox*2-3 spacer [~400bp] genetic markers. KA=*Kappaphycus alvarezii*; KS=*K. striatus*; KAr=*Kappaphycus* sp. "*Aring-aring*"; ED=*Eucheuma denticulatum*; 1kb =1 kilobase DNA ladder; -ve=negative control.

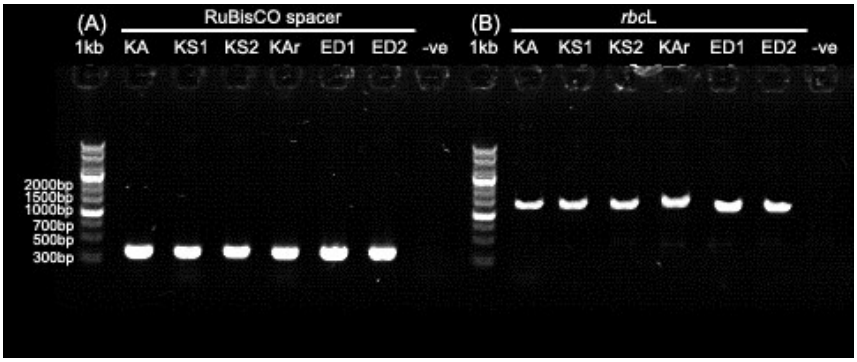

Figure 4.5: Electrophoretogram showing amplicons of the plastid **(**A**)** RuBisCO spacer [~400bp], **(**B**)** *rbc*L [~1,400bp] genetic markers. KA=*Kappaphycus alvarezii*; KS=*K. striatus*; KAr=*Kappaphycus* sp. "*Aring-aring*"; ED=*Eucheuma denticulatum*; 1kb=1 kilobase DNA ladder; -ve=negative control.

4.4 Data analysis

Sequencing results were satisfactory for all five molecular markers, where resulting electropherograms recorded good quality nucleotide peaks and little to non-existent noises. Contig assemblies were simple and direct, generating final sequences with no ambiguous nucleotides.

4.4.1 Molecular taxonomy and phylogenetics of *Kappaphycus* and *Eucheuma*

Phylogenetic reconstruction of *Kappaphycus* and *Eucheuma* specimens were performed for all five molecular markers tested in this dissertation. However, non-local specimens (i.e. samples from Philippines, Indonesia and Vietnam) collected in the present study were only incorporated into the *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L DNA marker datasets considering the poor phylogenetic resolution of the RuBisCO spacer. Results for each molecular marker are reported separately, as follows:

4.4.1.1 *cox*1

All 43 *cox*1 sequences (inclusive of GenBank records and outgroups) were easily aligned by eye, with no indels present. All sequences were truncated to a final length of 582 bp for phylogenetic comparisons with GenBank reference sequences. However, the full length (1,411 bp) of the *cox*1 sequences were deposited in GenBank (refer Table 4.1). *Hypnea charoides* H0923 (Yang et al. 2007) and *Gracilaria parvispora* G425 (Geraldino et al. 2006) were used respectively as outgroups for this particular dataset. The *cox*1 MSA block (excluding the outgroups) recorded 119 phylogenetically informative sites, 445 constant characters, and 18 phylogenetically uninformative characters.

Resulting phylogenetic trees (ML, MP and BI) were summarized and illustrated as a 50% majority-rule consensus maximum likelihood tree in Figure 4.6. Results

indicated a clear genetic difference between *Kappaphycus* and *Eucheuma* as compared to *Hypnea* and *Gracilaria* (ML=65.8%; MP=100%; BI=0.98). When the dataset was rooted with the outgroups, the *Kappaphycus* (Clade A-D) and *Eucheuma* (Clade E) clades were inferred to be monophyletic with supports of ML=72.7%; MP=73%; BI=0.82% and ML=99.9%; MP=64%; BI=1.00 respectively.

For *Kappaphycus*, the highly supported Clade A1 (ML=99.8%; MP=100%; BI=1.00) was composed mainly of *Kappaphycus* cultivars from South East Asia (Indonesia, Malaysia, Philippines and Vietnam) and a lone Hawaiian *Kappaphycus* 2614 (FJ554853). All local varieties of *K. alvarezii* were grouped together within Clade A1. On the other hand, Clade A3 was inferred with strong support (ML=99.4%; MP=100%; BI=1.00) to be monophyletic with solely *Kappaphycus* "*alvarezii*" specimens from Hawaii. *Kappaphycus striatus* specimens respectively formed two subclades B1 (ML=83.8%; MP=81%; BI=0.95) and B2 (ML=97.8%; MP=88%; BI=0.67), where the former clade consisted mostly of cultivated strains from SouthEast Asia; and the latter involved mostly wild Malaysian samples and a cultivated Philippine strain (*Kab-kab green*). Unable to resolve the phylogeny, the *cox*1 marker clustered *K. alvarezii* (Clade A1), the Hawaiian *K.* "*alvarezii*" (Clade A3) and *K. striatus* (Clade B) as a polytomy (ML=67.8%; MP=70%; BI=0.59). Local variety "*Aring-aring*" was shown to be genetically distinct from the other varieties, forming a monophyletic clade C sister to the aforementioned *Kappaphycus* congeners (ML=85.9%; MP=100%; BI=1.00). A lone *Kappaphycus cottonii* AOL186gz from the Philippines was shown to be sister (ML=72.7%; MP=73%; BI=0.82) to all other *Kappaphycus*. No *K. cottonii* specimens were collected from Malaysia in this study.

Species diversity of *Eucheuma* specimens collected in this study was low, where all specimens collectively formed Clade E (ML=99.6%; MP=64%; BI=1.00). One node within the clade (Clade E1) was mostly *E. denticulatum* "*Spinosum*" specimens from

Malaysia, Indonesia and the Philippines. These samples were also shown to be conspecific with the "*Spaghetti*" variety of *E. denticulatum* in the Philippines. "Clade" E1 consisted of the Malaysian "*Cacing*" variety, which grouped with an *E. denticulatum* "*endong*" variety from the Philippines and an *E. denticulatum* from Hawaii.

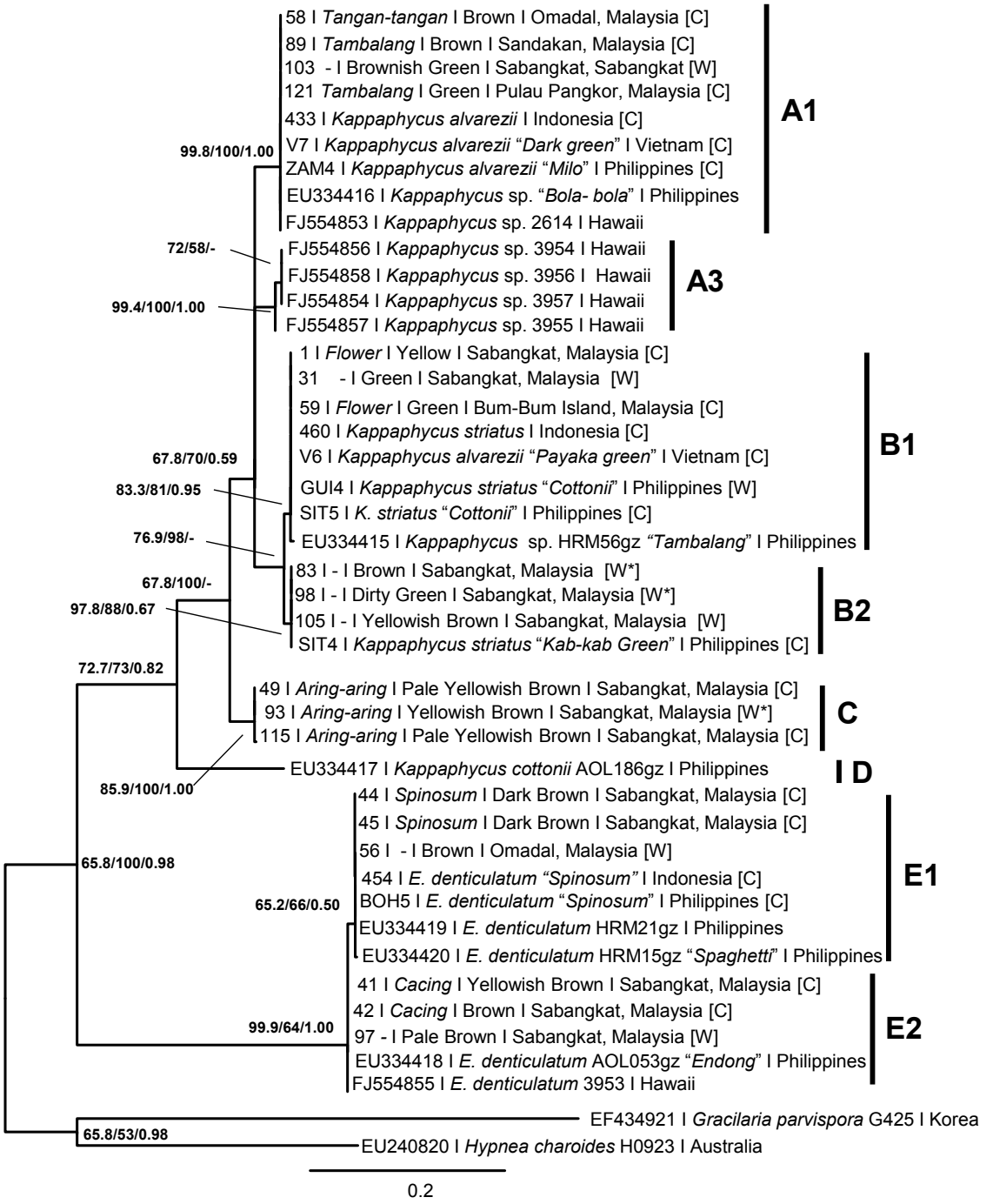

Figure 4.6: Maximum Likelihood 50% majority-rule consensus tree based on the *cox*1 genetic marker. -Ln likelihood score was -2111.89. (Substitution rate parameters: TC= 0.448703; TA= 0.072242; TG= 0.0123202; CA= $1.811302e^{-5}$; CG= 0.018013; AG= 0.448703). Nodal supports are arranged in an order of ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. Local specimens are denoted according to isolate no. | variety | color | origin | cultivated [C] or wild [W]. Non-local specimens were denoted as follows: isolate no. | sample name | | origin | cultivated [C] or wild [W]. Asterisks (*) indicate cystocarpic samples.

4.4.1.2 *cox*2

The *cox*2 dataset involves 31 sequences of 575bp in length. Considering the fact that this marker was newly designed, no other reference sequences were available in GenBank. *Gracilaria changii* (PSM122776-UMSS0696) and *Solieria* sp. (PSM12104-UMSS0259) were thus amplified to serve as the outgroups of the analysis. The *cox*2 sequences were easily aligned with few discrepancies. All *cox*2 sequences were submitted to GenBank (refer Table 4.1). The MSA block displayed 460 constant characters and 115 phylogenetically informative sites. All variable characters were reported to be phylogenetically informative.

Resulting phylogenetic trees (ML, MP and BI) were summarized and illustrated as a 50% majority-rule consensus maximum-likelihood tree in Figure 4.7. No reference sequences were available for the *cox*2 DNA marker in GenBank, and all sequences in the dataset are purely from this study. When rooted with the relatively more distant *Solieria* 120 and *Gracilaria changii* 98U samples, the *Kappaphycus* and *Eucheuma* specimens were respectively monophyletic with high support of ML=100%; MP=100%; BI=1.00 and ML=98.7%; MP=100%; BI=1.00. The tree topology was somewhat similar to that of the *cox*1 genetic marker, but with better resolution in general.

Cultivated *Kappaphycus alvarezii* specimens from Indonesia, Malaysia, Philippines and Vietnam were shown to be genetically similar to one another (Clade A1) with strong support (ML=100%; MP=100%; BI=1.00). *Kappaphycus* sp. 103 was also shown to be *K. alvarezii* despite being collected from the wild. Results have again showed that local variety names were not supported by molecular data. *Kappaphycus striatus* specimens were grouped together as Clade B (ML=86.6%; MP=98%; BI=1.00), and occurred as a sister group to *K. alvarezii* (Hawaiian *K. alvarezii* genotypes were not observed in this study). *K. striatus* samples were again clustered as two genotypes

(termed *K. striatus* B1/KS1 and *K. striatus* B2/KS2). Clade B1 consisted of mainly cultivated *K. striatus* from SouthEast Asia (ML=99.8%; MP=98%; BI=0.85), with two wild specimens from Malaysia (31) and the Philippines (GUI4). Wild specimens of *K. striatus* from Malaysia (83, 98 and 105) and a wild "*Kab-kab green*" sample from the Philippines were clustered together as Clade B with moderately high support (ML=99.8%; MP=79%; BI=0.87). Local "*Aring-aring*" varieties (Samples 49, 93and 115) were deduced as a different species from both *K. alvarezii* and *K. striatus* (ML=99.2%; MP=100%; BI=0.95) and occurred as a sister taxa to the said *Kappaphycus*. The cox2 genetic marker was able to further resolve samples 49 and 115 as being more genetically distinct from sample 93, thus positioned one node into Clade C (ML=78.8%; MP=63%; BI=0.62). No *Kappaphycus cottonii* samples were collected in this study.

The *Eucheuma denticulatum* specimens constitute Clade E1 and E2 respectively, where Clade E1 (ML=98.7%; MP=100%; BI=1.00) was made up of mainly the commercial "*Spinosum*" variety; whereas Clade E2 which occurred one node into Clade E1 were specimens of the "*Cacing*" variety (ML=95%; MP=92%; BI=0.96). No other Eucheumatoids were observed.

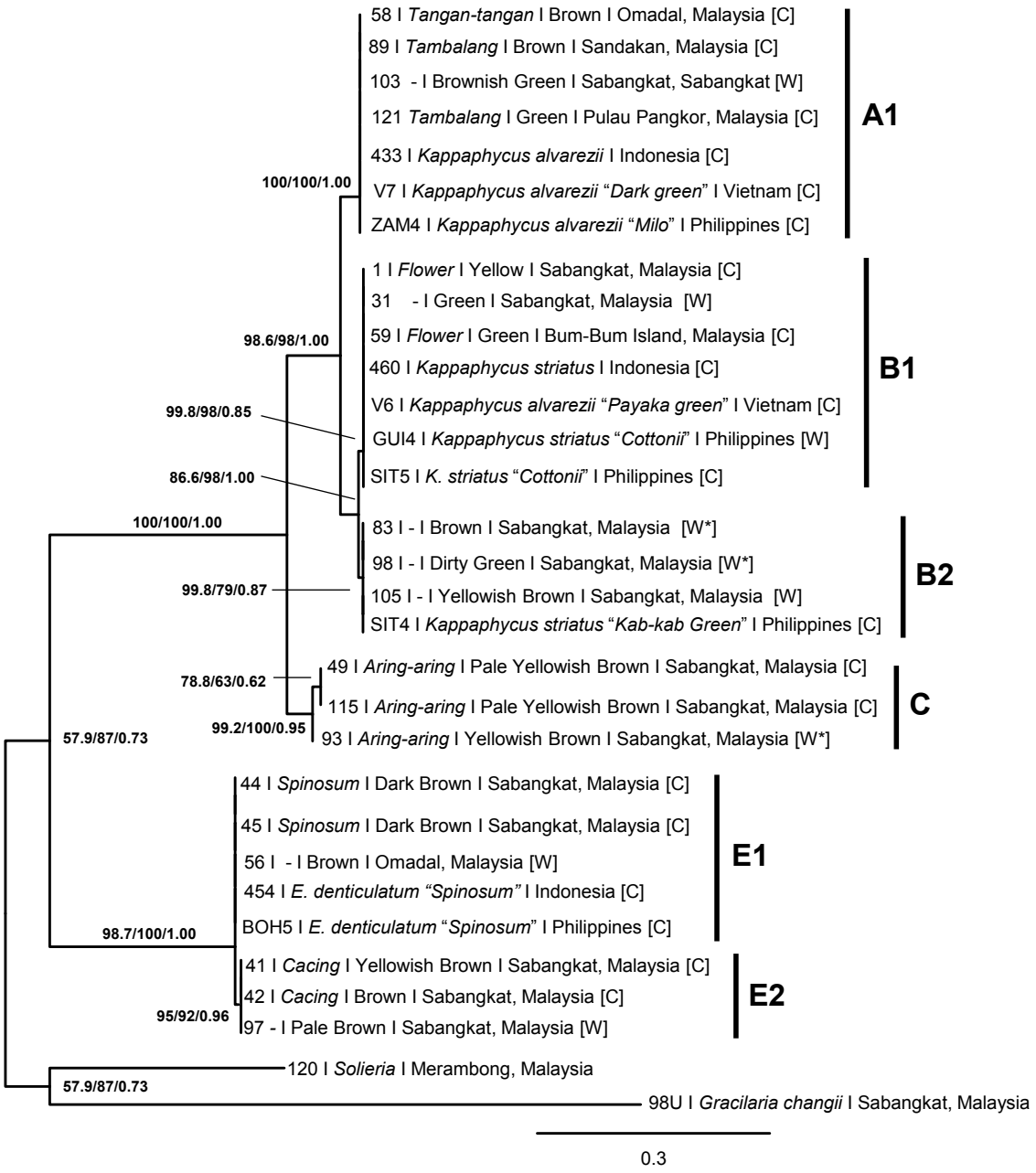

Figure 4.7: Maximum Likelihood 50% majority-rule consensus tree based on the *cox*2 genetic marker. -Ln likelihood score was -1745.14. (Substitution rate parameters: TC= 0.566112; TA= 0.001502; TG= 0.001502; CA= 0.064536; CG= 0.064536; AG= 0.301810). Nodal supports are arranged in an order of ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. Local specimens are denoted according to isolate no. | variety | color | origin | cultivated [C] or wild [W]. Non-local specimens were denoted as follows: isolate no. | sample name | origin | cultivated [C] or wild [W]. Asterisks (*) indicate cystocarpic samples.

4.4.1.3 *cox*2-3 spacer

The *cox*2-3 spacer, being the most commonly used genetic marker for *Kappaphycus* and *Eucheuma*, had numerous reference sequences in GenBank. Alignment of sequences was straightforward. A total of 76 sequences were used for the phylogenetic analysis, where sequence lengths were uniformly truncated, producing a MSA block of 341 bp in size. Adenine insertions were observed at positions 164 bp which were unique to African samples (E16, E130 and Reef4), and insertions of mostly Adenine and Thymine were frequent for non-*Kappaphycus* samples. All *cox*2-3 spacer sequences from this study were deposited into GenBank (refer Table 4.1). A *Solieria* sp. (PSM12104-UMSS0259) sample was assigned as the outgroup for this study because it was shown to produce better genetic resolution than the *Betaphycus philippinensis* used by Zuccarello et al. (2006). A total of 116 sites were regarded as phylogenetically informative, whereas 204 and 21 characters were respectively considered as constant and variable.

All resulting phylogenetic trees (ML, MP and BI) were again summarized as a 50% majority-rule maximum-likelihood consensus tree (Figure 4.8). Rooting of the entire *cox*2-3 spacer dataset with the *Solieria* outgroup resulted in two large clusters of samples- one composed of *Kappaphycus* sequences (Clade A-D), whereas the other mainly of *Eucheuma* sequences (Clade E-F), and a few *E. isiforme* (Clade G) and *Eucheuma* sp. E66. The phylogenetic resolution by the *cox2*-3 spacer marker was good, clearly distinguishing among species. By omitting singleton sequences, the *Kappaphycus* clade was inferred to be monophyletic with high support (ML=100%; MP=100%; BI=1.00). The delineation of *Eucheuma* species was relatively obscure, with no clear indication of monophyly- Clade E was composed mainly of *E. denticulatum* specimens (ML=100%; MP=100%; BI=1.00) whereas Clade F consisted of *E. platycladum* and several *Eucheuma* sp. (ML=96.1%; MP=81%; BI=1.00), but there was

no support that Clades E and Clades F were monophyletic. The basic tree topology of the cox2-3 spacer phylogenetic tree was similar to that of the *cox*1 and *cox*2 trees.

Considering the large amounts of reference sequences from GenBank, the *Kappaphycus* clade was composed of four smaller clades A to D. Clade A1 (ML=84.7%; MP=79%; BI=0.80) was comprised mainly of *K. alvarezii* cultivated throughout the globe- Columbia, Hawaii, Indonesia, Malaysia, Panama, Philippines, Africa and Vietnam (Conklin et al. 2009; Halling et al. 2012; Zuccarello et al. 2006). Three *K. alvarezii* samples E130, Reef4 and E16 from Tanzania, Paje-Jambiani and Madagascar respectively were clustered together as Clade A2 (ML=85.3%; MP=73%; BI=1.00), which occurs as a sister taxa to Clade A1. Four Hawaiian *Kappaphycus* "*alvarezii*" (E57, E71, 919 and 3955) were shown to be genetically different from members of Clade A1 as well as A2 and monophyletic, constituting Clade A3 with high support (ML=99.8%; MP=98%; BI=1.00). However, similar to that observed in the earlier *cox*1 phylogenetic tree, the taxonomic position of Clade A3 was uncertain, and remained in polytomy with both *K. alvarezii* (Clade A1 and A2) and *K. striatus* (Clade B1 and B2). Commercially cultivated strains of *K. striatus*, and a wild Philippine sample (GUI4) were all grouped within Clade B1 with low to moderate support of ML=78.6%; MP=70%; BI=0.54. Local *K. striatus* varieties were all members of Clade B1 regardless of color and morphological differences. The monophyly of Clade B2 which was composed largely of *K. striatus* collected from the wild, was poorly supported (ML=54%; MP=-%; BI=0.57). A cultivated sample of this particular species was also reported from the Philippines (SIT4), along with two unpublished sequences (D13 and D14) from Malaysia. All these *Kappaphycus* species were resolved as a sister group to the local "*Aring-aring*" varieties, be it cultivated or wild ones, with high support, indicating the difference in terms of genetic composition (ML=99.8%; MP=93%; BI=0.80). Due to the lack of confidence,

the lone *Kappaphycus cottonii* E108 from the Philippines remained unresolved by the *cox*2-3 spacer DNA marker.

E. denticulatum samples were all clustered together, forming three subclades E1, E2 and E3 (ML=100%; MP=100%; BI=01.00). Members of Clade E1 were mostly commercially cultivated "*Spinosum*" seaweeds that are prevalent in SouthEast Asia and also Hawaii. Clade E2 was comprised of *E. denticulatum* specimens which were inferred with low support (ML=58.6%; MP=77%; BI=0.63) to be genetically dissimilar to that of *E. denticulatum* of Clade E1. Malaysian "*Cacing*" varieties fall into this particular Clade, along with two Hawaiian *E. denticulatum* (888 and 3953). Although also named *E. denticulatum*, members of Clade E3 (ML=98.6%; MP=100%; BI=1.00) occurred as a sister taxa to Clade E1 and E2 (ML=100%; MP=100%; BI=1.00), and were exclusive to Africa. Clade F (ML=96.1%; MP=81%; BI=1.00) consisted of two bifurcations into two subclades, where one was resolved to be monophyletic with African *E. platycladum* specimens (ML=100%; MP=100%; BI=1.00), whereas the other subclade were made up of unidentified *Eucheuma* samples. The taxonomic position of *Betaphycus philippinensis* E118 was unresolved within the *cox*2-3 spacer dataset. Clade G, the basal group of the phylogenetic tree, was composed of *Eucheuma isiforme* specimens (E2, E35 and E37) which collectively occur as a sister group to all the aforementioned *Kappaphycus*, *Eucheuma* and *Betaphycus* samples (ML=100%; MP=100%; BI=1.00). This had rendered *Eucheuma* specimens paraphyletic within this particular *cox2*-3 spacer dataset.

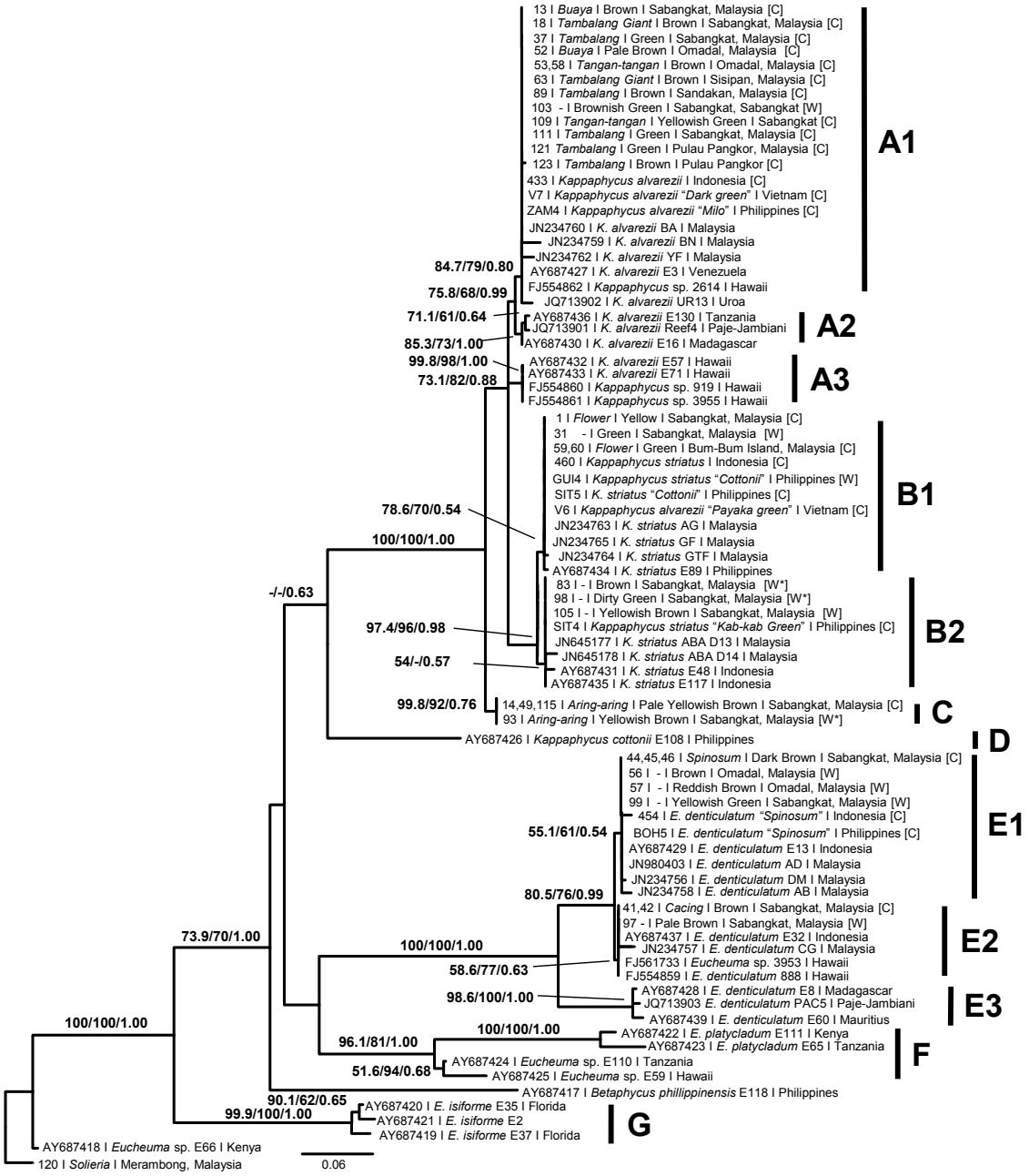

Figure 4.8: Maximum Likelihood 50% majority-rule consensus tree based on the *cox*2-3 spacer genetic marker. -Ln likelihood score was -2051.78. (Substitution rate parameters: TC= 0.451710; TA= 0.009195; TG= 0.019224; CA= 0.066568; CG= 0.001594; AG= 0.451710). Nodal supports are arranged in an order of ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. Local specimens are denoted according to isolate no. | variety | color | origin | cultivated [C] or wild [W]. Non-local specimens were denoted as follows: isolate no. | sample name | origin | cultivated [C] or wild [W]. Asterisks (*) indicate cystocarpic samples.

4.4.1.4 *rbc*L

A total of 45 *rbc*L sequences were used, encompassing a length of 1,466bp. All *rbc*L sequences from this studies were successfully deposited in GenBank (refer Table 4.1). For shorter GenBank sequences, ambiguous "N" nucleotides were added to make up the final and uniform length. Sequence alignment was direct, with little inconsistencies. No indels were present among the *Kappaphycus* and *Eucheuma* specimens. Of the 1,466bp MSA block, 225 characters were reported as being informative phylogenetically, 1052 characters constant, and 190 variable characters lacking phylogenetic information.

A 50% majority-rule maximum-likelihood consensus tree was constructed with support values compiled from the ML, MP and BI analyses respectively, depicted as Figure 4.9. The *Solieria* and *Gracilaria* outgroups utilized in this particular dataset were similar to that of *cox*2. When rooted, the genus *Kappaphycus* was resolved as monophyletic with high support (ML=100%; MP=88%; BI=1.00). However this was not the case for *Eucheuma* samples; the Eucheumatoids were scattered throughout the basal part of the phylogenetic tree, displaying a paraphyletic nature, much like that observed for the *cox*2-3 spacer. Despite the inconsistencies within the tree, taxonomic grouping of samples of this study coincide with results based on other genetic markers.

Commercially farmed *Kappaphycus alvarezii* were all grouped together within Clade A1 with strong support (ML=85.2%; MP=100%; BI=1.00). These include several *K. alvarezii* species from the Philippines, where two (1999C and 1999N) were shown to be slightly different genetically from the rest, occurring one node into Clade A1 with a support of ML=97%; MP=98%; BI=1.00. Molecular data has similarly showed that different local varieties are actually conspecific. No Hawaiian sequences were available for the *rbc*L marker, and thus the phylogenetic tree is devoid of Clade A3. Due to that,

Kappaphycus striatus (Clade B) was shown to be the direct sister group of *K. alvarezii* (ML=96.7%; MP=88%; BI=1.00), with two subclades B1 and B2. Commercial *K. striatus* strains, including a "*Sacol*" variety and two probably misidentified *Kappaphycus* "*cottonii*" from the Philippines, constituted Clade B1 with inconsistent support (ML=77.2%; MP=58%; BI=0.99). "Clade" B2 was not supported in this tree. However, the three *K. striatus* collected from the wild and a cultivated Philippine "*Kab-kab Green*" variety were shown to be in a polytomy sister to Clade B1 (ML=90.3%; MP=100%; BI=1.00). The plastid *rbc*L marker has also resolved the "*Aring-aring*" varieties as a sister group to both the *K. alvarezii* and *K. striatus* (ML=100%; MP=88%; BI=1.00). A lone *Eucheuma arnoldii* (AF099690) was surprisingly positioned as sister to all the *Kappaphycus* congeners with strong support (ML=83%; MP=100%; BI=1.00).

The taxonomy of *Eucheuma* was poorly resolved using the *rbc*L molecular marker. However, the phylogenetic construction associated with *Eucheuma* samples collected in this study was acceptable, where samples of the *E. denticulatum* "*Spinosum*" variety were all clustered together within Clade E1 (ML=100%; MP=100%; BI=1.00), whereas those of the "*Cacing*" variety formed Clade E2 (ML=99.9%; MP=88%; BI=0.73). No other GenBank *E. denticulatum* sequences were available at this time. Clade E was inferred to form a sister group to a lone *Eucheuma serra* sample from Taiwan with strong support (ML=97.3%; MP=91%; BI=1.00). Although two *Betaphycus* specimens (AF099684 and AF099685) were available from the Philippines, their phylogenetic relationship to *Kappaphycus* and other *Eucheuma* are unclear. Much like that observed for the *cox*2-3 spacer, a lone *Eucheuma isiforme* and an additional *E. uncinatum* sample were isolated from the rest of the *Eucheuma* specimens, rendering the genus paraphyletic.

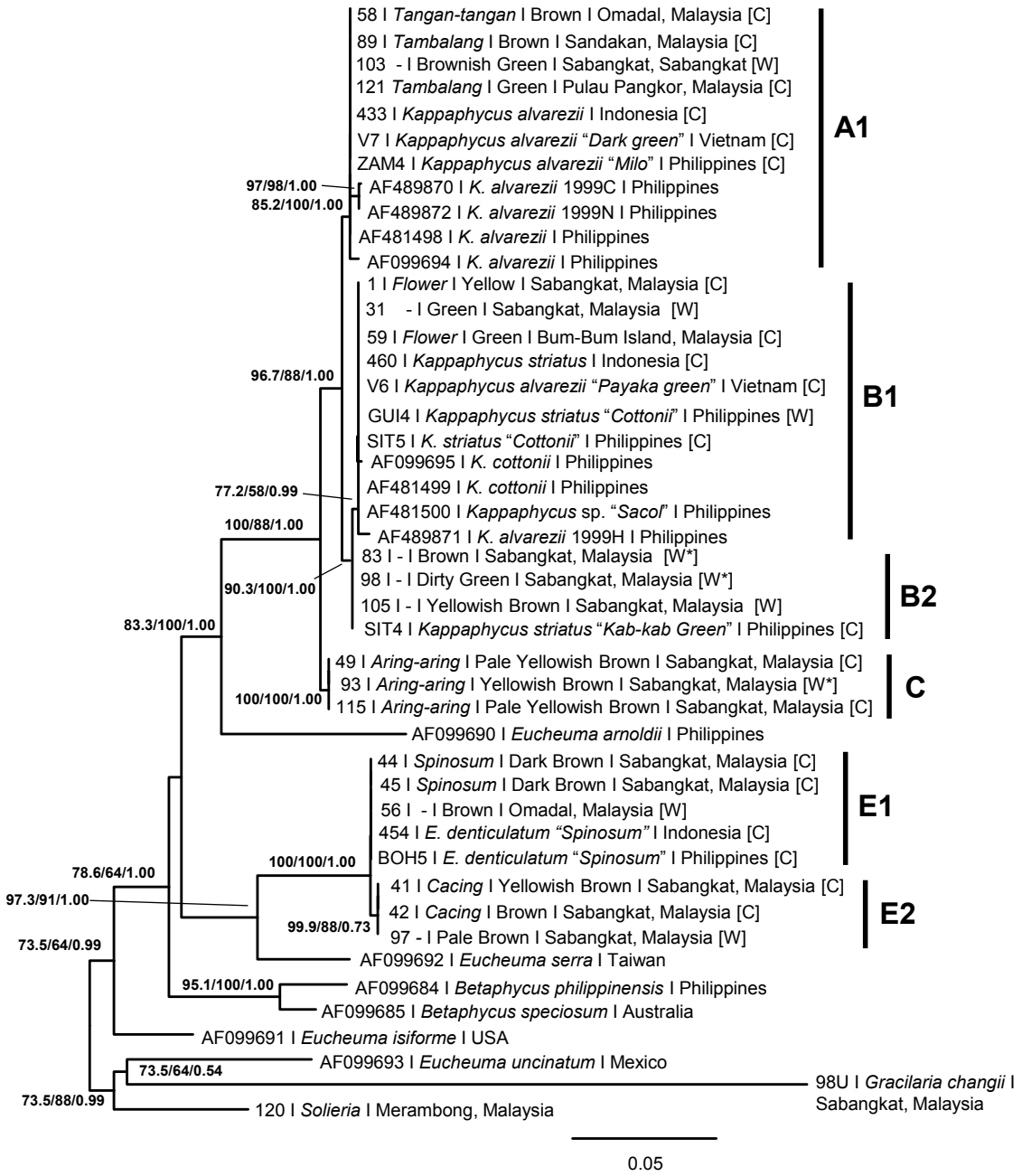

Figure 4.9: Maximum Likelihood 50% majority-rule consensus tree based on the *rbc*L genetic marker. -Ln likelihood score was -5287.27. (Substitution rate parameters: TC= 0.609655; TA= 0.063279; TG= 0.026625; CA= 0.026625; CG= 0.0.63279; AG= 0.210535). Nodal supports are arranged in an order of ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. Local specimens are denoted according to isolate no. | variety | color | origin | cultivated [C] or wild [W]. Non-local specimens were denoted as follows: isolate no. | sample name | origin | cultivated [C] or wild [W]. Asterisks (*) indicate cystocarpic samples.

4.4.1.5 RuBisCO spacer

Although reported to offer less resolving power as compared to the *cox*2-3 spacer (Conklin et al. 2009; Zuccarello et al. 2006), the RuBisCO spacer is still the second most extensively used genetic marker for *Kappaphycus* and *Eucheuma*. A total of 53 RuBisCO spacer sequences were compiled within the dataset, which includes GenBank sequences. Sequences were again easily aligned, with apparent Thymine insertions at position 78bp for a majority of *Eucheuma* specimens. Due to the limited resolution capability, a *Solieria* outgroup did not work well for the RuBisCO spacer dataset; instead, *Eucheuma isiforme* samples were utilized. The genus *Eucheuma* was shown to be paraphyletic with respect to *Betapyhcus philippinensis* by Zuccarello and co-workers (2006) (also supported by the *cox*2-3 spacer and *rbc*L markers of this study), where *E. isiforme* occurred as a sister taxa to *Kappaphycus*, *E. denticulatum*, *E. platycladum* and several *Eucheuma* sp. RuBisCO spacer sequences generated in this study were submitted to GenBank (refer Table 4.1). The final MSA sequence block was 264bp in size, with 44 phylogenetically informative sites, and 13 variable characters.

ML, MP and BI results were integrated and displayed as a 50% majority-rule consensus maximum-likelihood tree in Figure 4.10. The RuBisCO spacer was not very capable of resolving the phylogeny of *Kappaphycus* and *Eucheuma*, with no support for the monophyly of both genera. However, when *Kappaphycus cottonii* specimens were disregarded, the remaining *Kappaphycus* were resolved with moderate to strong support (ML=74.1%; MP=85%; BI=0.98). The RuBisCO spacer marker displayed relatively weak resolution power, with limited specificity in terms of species delineation.

All common *K. alvarezii* cultivars were clustered together in polytomy, designated “Clade” A with moderate support only under the Maximum Likelihood criteria (ML=71.2%). The genetic distinctiveness of *K. alvarezii* E16 from Madagascar

was not clear in the RuBisCO spacer phylogenetic tree (as opposed to that shown by the *cox*2-3 spacer). Occurring as a sister taxa to these *K. alvarezii* specimens were *K. striatus* specimens of Clade B (ML=94.1%; MP=87%; BI=1.00), a Hawaiian *K. alvarezii* E57 and a lone Philippine *Kappaphycus* "*Bola-bola*" variety. The genetic assortment of these samples disagree with tree topologies by the other four genetic markers, were *K. striatus* consisted of two genotypes B1 and B2. The Hawaiian *K. alvarezii* E57 was shown to be more closely related to *K. striatus* with low support (ML=62.5%; MP=-%; BI=0.66). The local "*Aring-aring*" varieties were monophyletic (ML=86.8%; MP=61%; BI=0.89), and occur as a sister taxa to both *K. alvarezii* and *K. striatus* (ML=74.1%; MP=85%; BI=0.98). Although there is no support, *Kappaphycus cottonii* specimens were inferred to be more closely related to the *Eucheuma* by the RuBisCO spacer.

The taxonomic reconstruction of *Eucheuma* specimens for the RuBisCO spacer dataset is confusing, with no clear clustering of specimens. However, an apparent pattern of grouping was observed for *E. denticulatum* specimens with high support (ML=99.7%; MP=100%; BI=1.00). Although the RuBisCO spacer DNA marker was not capable of delineating between the "*Spinosum*" and "*Cacing*" varieties as the *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L markers did, it was able to genetically differentiate the *E. denticulatum* E60 exclusive to Africa from the rest. Clade F (ML=61.5%; MP=68%; BI=1.00) comprised of *E. platyclaudum* (E65 and E111) from Africa and also two unidentified *Eucheuma* specimens coincide with that observed for the *cox*2-3 spacer. The RuBisCO spacer resolved *Betaphycus philippinensis* E118 from the Philippines as a sister group to Clade F with moderate to high support (ML=64.3%; MP=75%; BI=0.90). Clade G was composed of *Eucheuma isiforme* specimens (ML=99.6%; MP=99%; BI=1.00).

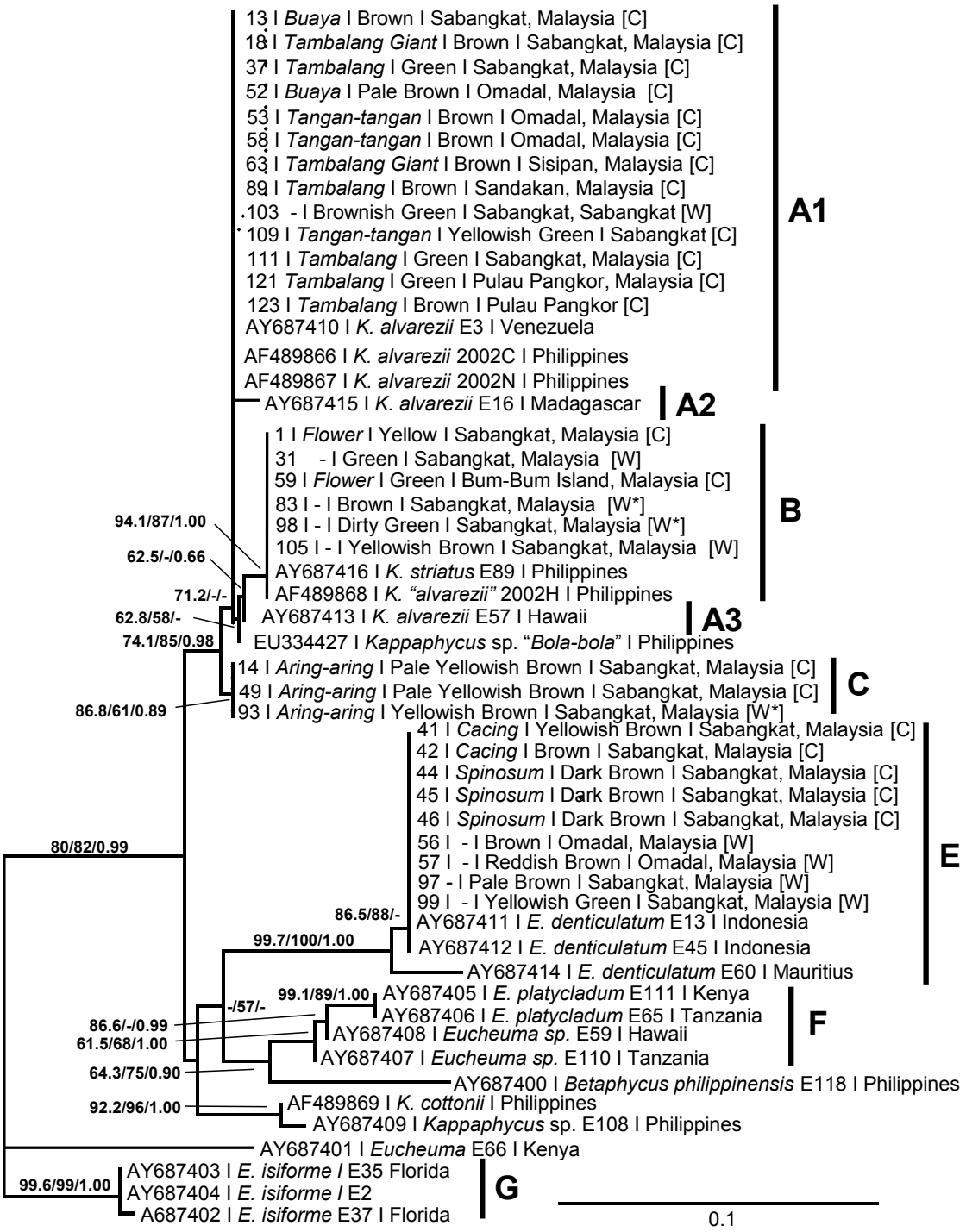

Figure 4.10: Maximum Likelihood 50% majority-rule consensus tree based on the RuBisCO genetic marker. -Ln likelihood score was -795344. (Substitution rate parameters: TC= 0.536556; TA= 0.007765; TG= 0.0071856; CA= 0.071856; CG= 0.007765; AG= 0.304203). Nodal supports are arranged in an order of ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. Local specimens are denoted according to isolate no. | variety | color | origin | cultivated [C] or wild [W]. Non-local specimens were denoted as follows: isolate no. | sample name | origin | cultivated [C] or wild [W]. Asterisks (*) indicate cystocarpic samples.

4.4.1.6 Haplotype analyses

The *cox*2-3 spacer genetic region was chosen as the basis to infer gene genealogies for *Kappaphycus* and *Euchuema* at the population level due to the large number of GenBank reference sequences. The resulting haplotype networks were generated with a connection limit of 11 steps for *Kappaphycus* (Figure 4.11), and 30 steps for *Eucheuma* (Figure 4.12). Representative haplotype annotations followed those outlined in earlier studies (Halling et al. 2012; Zuccarello et al. 2006).

Most of the *Kappaphycus alvarezii* samples collected in the present study exhibit haplotype 3 along with counterparts from all over the globe. Two new haplotypes from Malaysia (unpublished data), namely YF (JB234762) and BN (JN234759) were shown to be one and three base pairs different from hapolotype 3. No haplotypes similar to those recorded from Africa (16, 130, Reef4 and UR13) were observed. Two Hawaiian samples (919 and 3955) by Conklin and co-workers (2009) recorded eight base pairs difference from haplotype 3, and were similar to haplotypes 57 and 71 (Zuccarello et al. 2006). *K. striatus* haplotypes 89 and 117 of nine and five samples respectively were observed in the present study. Two new haplotypes from GenBank, AG (JN234763) and GTF (JN234764), from Malaysia were observed, both with one base pair difference from haplotype 89. Haplotype 89 is separated from haplotype 117 by one hypothetic haplotype; whereas haplotype 117 is different from haplotypes 48 and a new Malaysian haplotype D14 (unpublished data, JN645178) by one nucleotide. This D14 haplotype was computed to be eleven base pair different from haplotype 3. *Kappaphycus* sp. "*Aring-aring*" consisting of three Malaysian samples were revealed to be twelve base pairs different from *K. alvarezii* haplotype 3 as well, yet showed no affinity to existing haplotypes, and was hence assigned as a new haplotype MY14.

Most of the *E. denticulatum* samples collected in this study (n=9) belong to the *E. denticulatum* "*Spinosum*" haplotype 13. Conklin et al. (2009) reported two Hawaiian *E. denticulatum* that resembled haplotype 32, with three base pair difference from haplotype 13. The Malaysian "*Cacing*" varieties (41, 42, and 97) were also clustered together within haplotype 13. Sample 454 from Kertasari (Indonesia) was assigned as a new haplotype which is one base pair different from haplotype 13, and four nucleotides different from haplotype 32. Two new GenBank haplotypes from Malaysia were also established, namely CG (JN234757) and DM (JN234756). The former is three base pairs different from haplotype 32, whereas the latter is one nucleotide different from haplotype 13. No haplotype match was observed for the African haplotypes 8, 60 and PAC5 (Halling et al. 2012; Zuccarello et al. 2006).

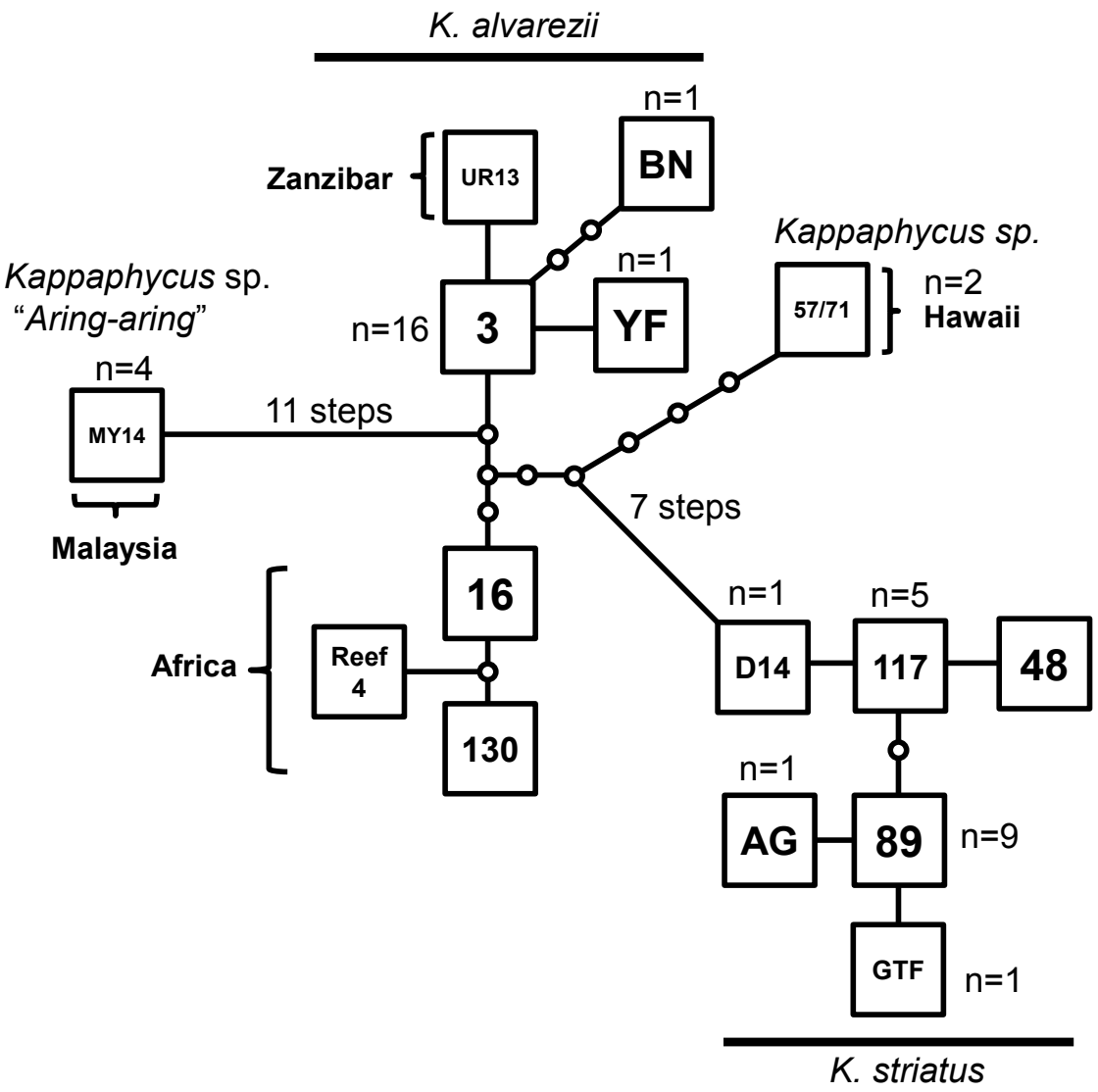

Figure 4.11: Haplotype networks for *Kappaphycus* samples based on the mitochondrial *cox*2-3 spacer. Haplotype annotation and nomenclature are based on Halling et al. (2012) and Zuccarello et al. (2006) with minor modifications. Each line represents a point mutation whereas empty circles indicate hypothetical haplotypes. *n*= number of samples (only sequences not reported in previous studies were counted). *Kappaphycus cox*2-3 spacer haplotypes: 3 (*n*= 13, 18, 52, 53, 58, 63, 89, 103, 109, 121, 123, 433, 2614, BA, V7, ZAM4); 89 (*n*= 1, 31, 59, 60, 460, GF, GUI4, SIT5, V6); 117 (*n*= 83, 98, 105, SIT4, ABA_D13); 57/71 (*n*= 919, 3955); MY14 (*n*= 14, 49, 93,115).

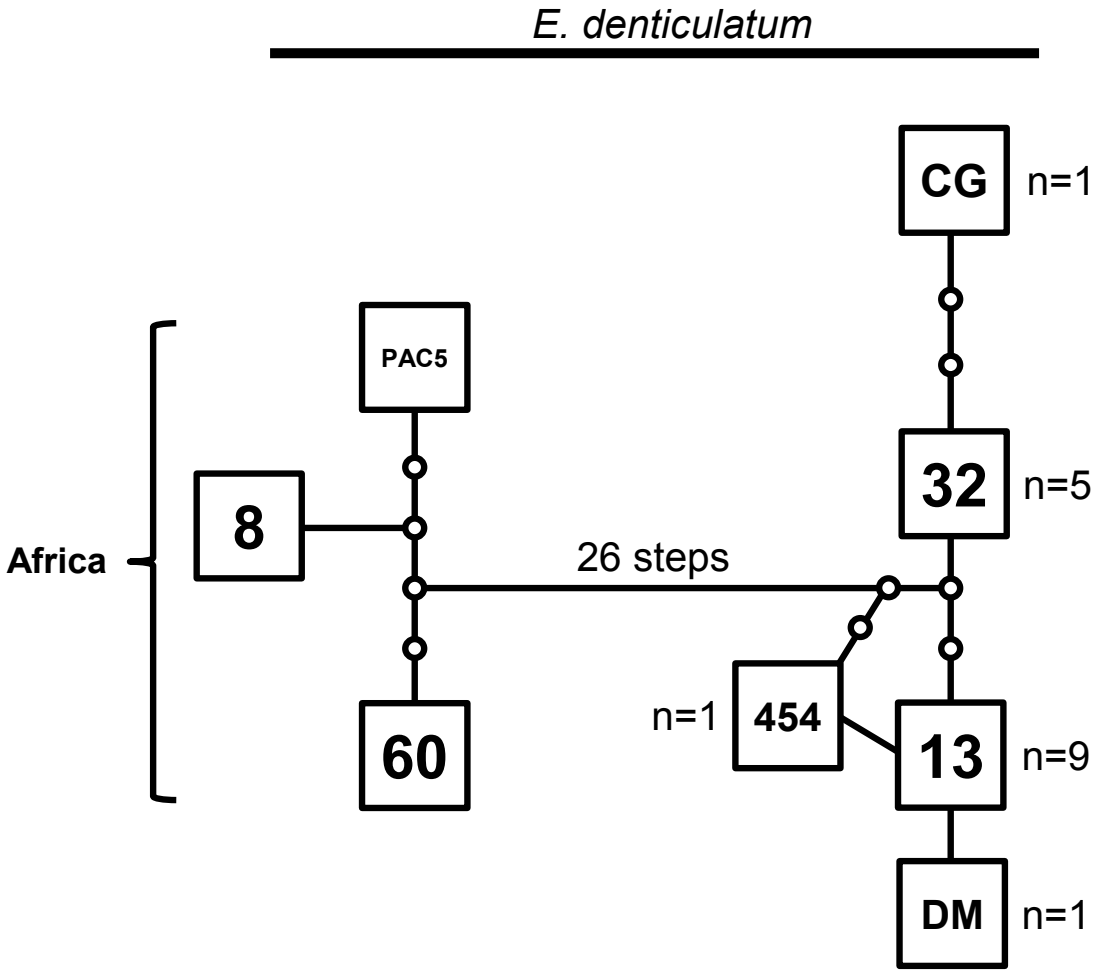

Figure 4.12: Haplotype networks for *Eucheuma denticulatum* samples based on the mitochondrial *cox*2-3 spacer. Haplotype annotation and nomenclature are based on Halling et al. (2012) and Zuccarello et al. (2006) with minor modifications. Each line represents a point mutation whereas empty circles indicate hypothetical haplotypes. *n*= number of samples (only sequences not reported in previous studies were counted). *Eucheuma cox*2-3 spacer haplotypes: 13 (*n*= 44, 45, 46, 56, 57, 99, AB, AD, BOH5); 32 (*n*= 41, 42, 97, 888, 3953).

4.4.2 Molecular marker assessment

4.4.2.1 Distance-based DNA identification criteria

Distance-based DNA identification criteria on the *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L molecular markers for 29 selected *Kappaphycus* and *Eucheuma* samples from Southeast Asia were carried out using TaxonDNA (Meier et al. 2006). Plots of pairwise distances based on the corrected Kimura 2-parameter (K2P) are illustrated as Figure 4.13. Apart from *cox*1, the intra- and interspecific genetic distances showed identical results for the *cox*2, *cox*2-3 spacer and *rbc*L genetic markers when computed using (i) the corrected K2P and (ii) uncorrected pairwise distances. *cox*1 recorded the largest distance (K2P: 0.64% for total overlaps and 0.71% for 90% overlaps; uncorrected pairwise distance: 0.63% for total overlaps and 0.70% for 90% overlaps) between the smallest pairwise distance among interspecific but intrageneric samples and the largest pairwise distance among intraspecific sequences (also known as a "Barcoding Gap"). This was followed by *cox*2 (0.52% for total and 90% overlaps), *cox*2-3 spacer (0.27% for total and 90% overlaps), and finally *rbc*L (0.06% differences for total and 90% overlaps), with the smallest "Barcoding Gap".

Identification successes based on the distance-based Best Match (BM) and Best Close Match (BCM) models were summarized as Figure 4.14. All four genetic markers were capable of correctly identifying all 29 specimens within the dataset under the BM criteria. For the BCM criteria, *rbc*L showed the highest success in identification with 100%, followed by *cox*2 (96.6%), *cox*2-3 spacer (93.1%) and *cox*1 (79.3%). No matches were recorded at values 3.44%, 6.89% and 20.68% for *cox*2, *cox*2-3 spacer and *cox*1 respectively.

Genetic distances of *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L genetic markers are tabulated as Appendix H-K.

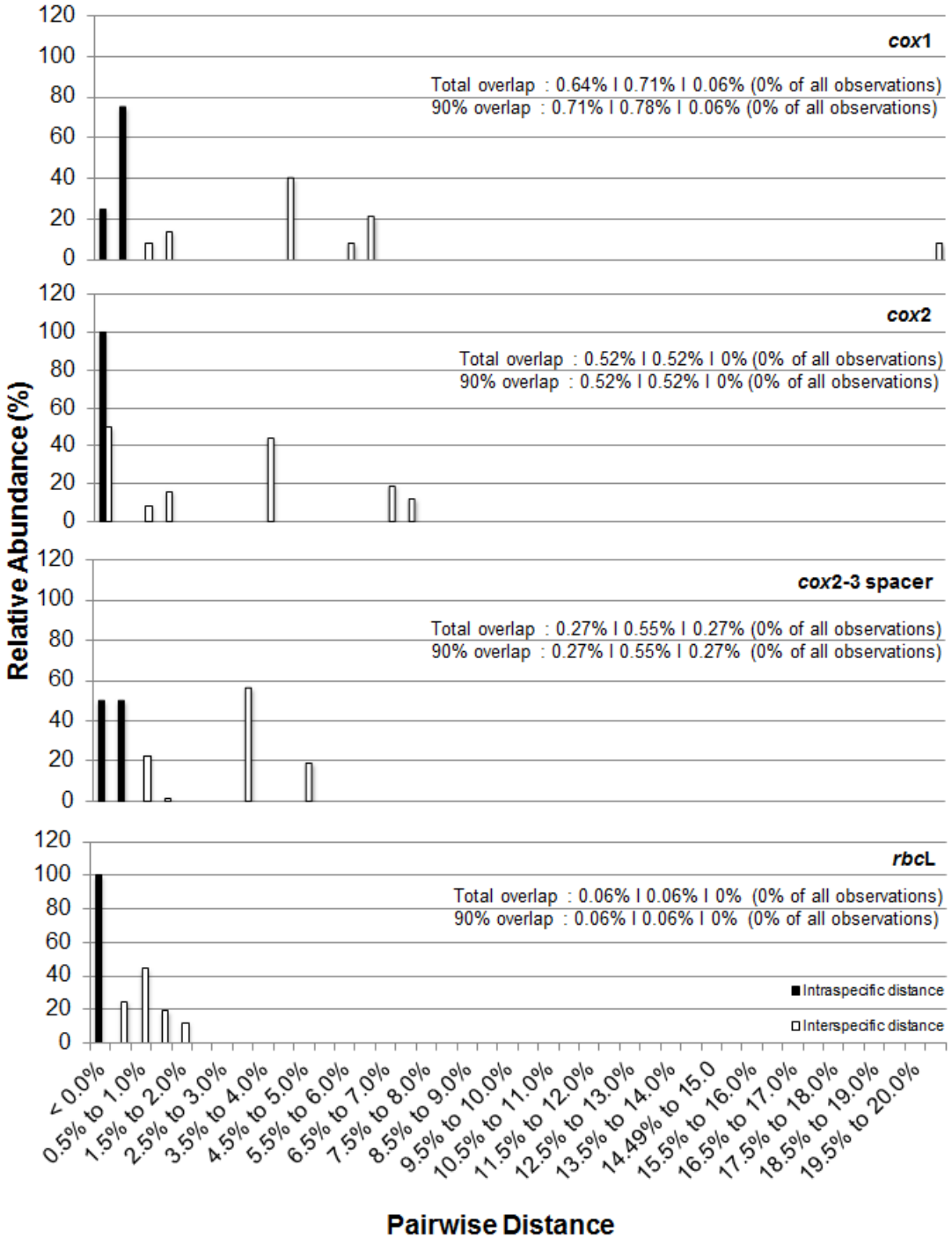

Figure 4.13: Plot of intra- and interspecific genetic distances for the *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L DNA markers. Numeric values are arranged according to: the difference between the smallest interspecific but intrageneric distance and the largest intraspecific distance | smallest pairwise distance between interspecific but intrageneic sequences | largest pairwise distance between intraspecific sequences; followed by the number of observations affected (in brackets).

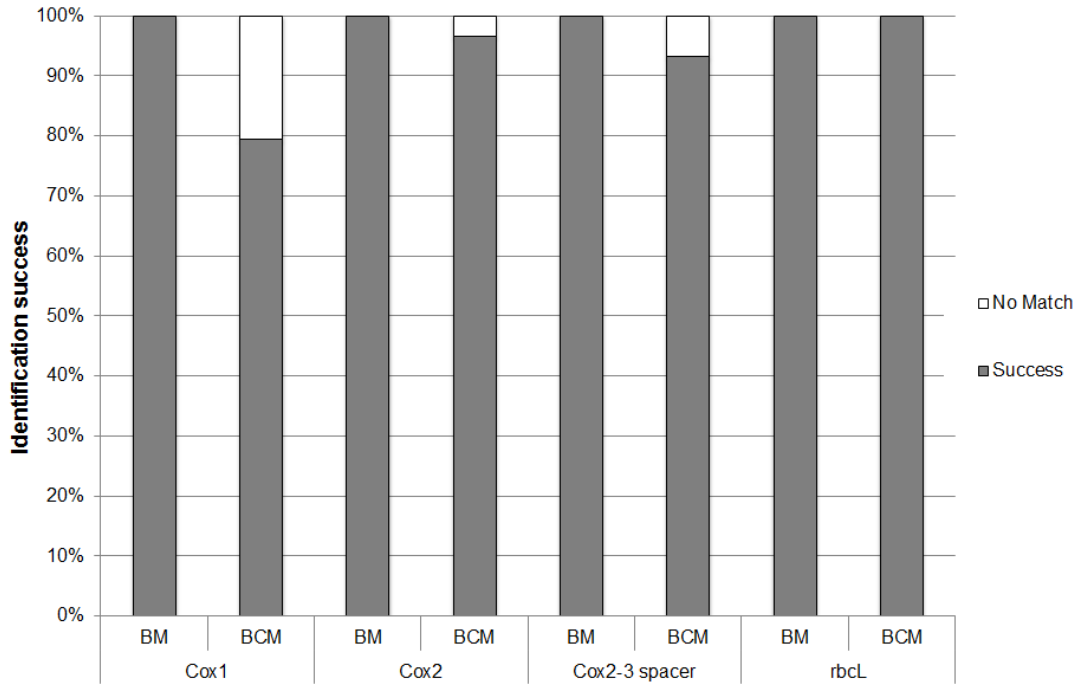

Figure 4.14: Identification success of the *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L DNA markers. Results were computed based on the *Best Match* (BM) and *Best Close Match* (BCM) criteria.

4.4.2.2 Tree-based DNA identification criteria

Resulting NJ trees for the *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L molecular markers are depicted as Figure 4.15. Specimens were annotated with merely their codes for easier referencing (details available in Table 4.1). ML, MP and BI phylogenetic trees with similar tree topologies were provided as Appendix O-R. Although nodal support values may vary, all trees resolved *Kappaphycus* and *Eucheuma* as two distinctive, monophyletic clades, with one Operational Taxonomic Unit (OTU) for *K. alvarezii* (KA1), two OTUs for *K. striatus* (KS1 and KS2), one OTU for the local "*Aring- aring*" variety (KAr) and two OTUs for *E. denticulatum* (ED1 and ED2). The application of Hebert et al.'s (2003a) tree-based identification saw 100% identification success across all species for every molecular marker. However, apart from the *rbc*L marker, identification accuracy decreased when measurements were made using the criteria by

Meier et al. (2006), where the *cox*1 recorded a drop to 89.7%, whereas both the *cox*2 and *cox*2-3 spacer decreased to an identification success rate of 96.6%. Sequences that were not successfully identified were categorized as ambiguous.

Despite the equal ability of all four markers to cluster species consistently and somewhat accurately, the *cox*1 genetic marker displayed more specific resolution in terms of intraspecific genetic variations, whereas the *rbc*L displayed the opposite, with relatively low genetic variability on both the intraspecific and interspecific level.

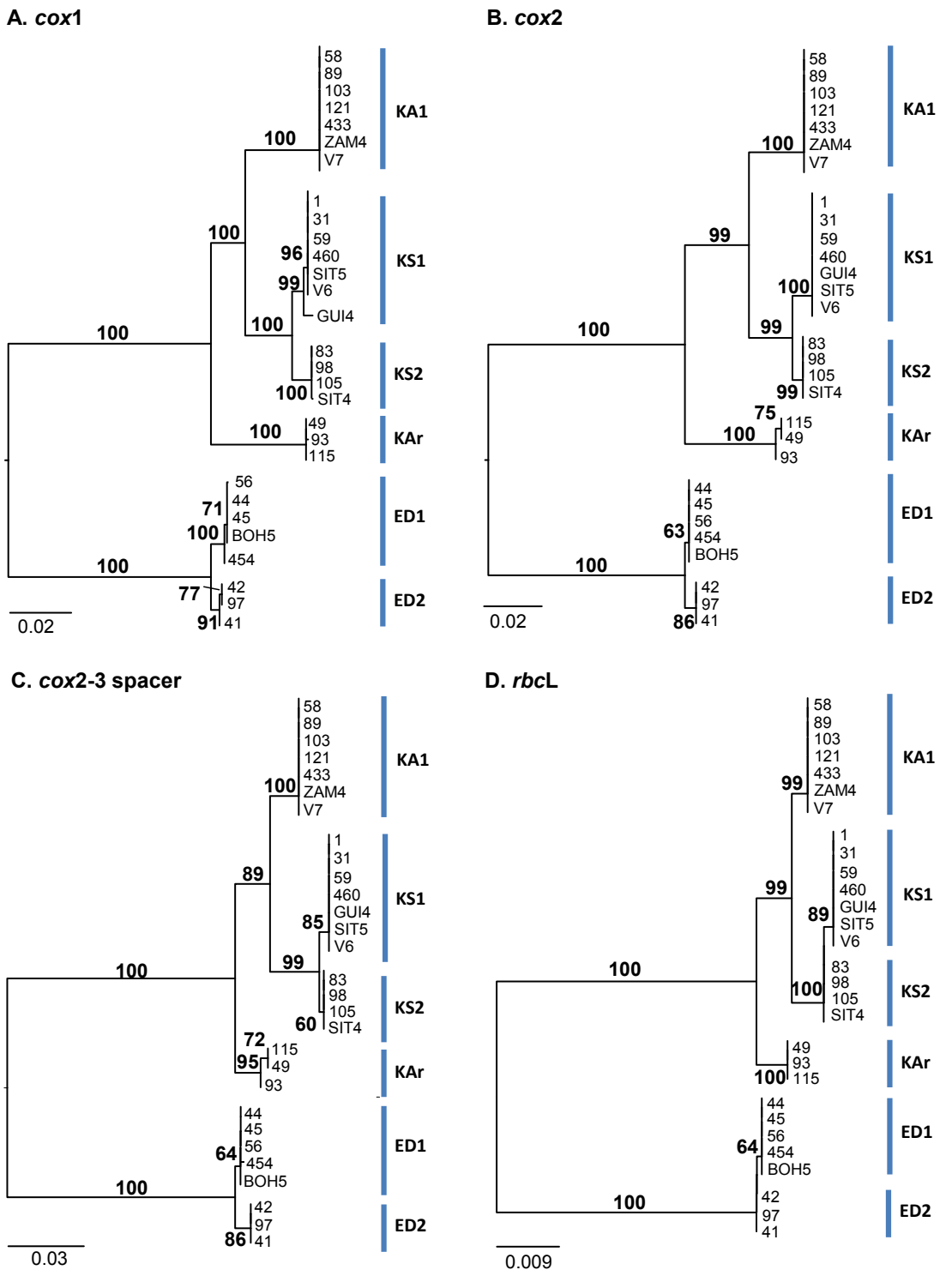

Figure 4.15: Neighbour Joining (NJ) trees of selected *Kappaphycus* and *Eucheuma* from Southeast Asia based on (A) *cox*1; (B) *cox*2; (C) *cox*2-3 spacer; (D) *rbc*L molecular markers. Numbers at node indicate corresponding bootstrap values over 1,000 replicates. Clade annotations represent Operational Taxonomic Units (OTU), where KA= *Kappaphycus alvarezii*; KS= *Kappaphycus striatus*; KAr = *Kappaphycus* sp. "*Aringaring*"; ED= *Eucheuma denticulatum*.

4.4.3 Large dataset assessment

The mitochondrial encoded *cox*2-3 spacer, which is to date the most extensively used genetic marker for *Kappaphycus* and *Eucheuma* was selected as the DNA barcode for Large Dataset Assessment. The dataset utilized was exactly the same as the one mentioned in Section 4.4.1.3, apart from the inclusion of the *Solieria* outgroup. Samples were grouped according to two taxonomic assortments consisting of either ten Operational Taxonomic Units (OTU) or five non-OTUs (Table 4.1).

4.4.3.1 Distance-based DNA identification criteria

No differences were observed when the *cox*2-3 spacer dataset was analyzed for pairwise distance under the uncorrected and K2P corrected distances. Figure 4.16 illustrates the intraspecific and interspecific genetic divergence based on the K2P model between Operational Taxonomic Unit (OTUs) and non-OTUs. For the former, a pairwise distance overlap of 0.29% was observed between the smallest interspecific and the largest intraspecific sequences; whereas no overlaps were observed for the latter.

Identification successes based on the BM and BCM criteria are shown in Figure 4.17. All species were correctly identified under BM for OTU and non-OTU analyses. Under BCM selection, 90.66% of samples were correctly identified when species were considered as OTUs, and 97.33% when species were not considered as OTUs. No matches were found for the remaining queries. Under the *All Species Barcodes* (ASB) identification criteria, an identification success of 90.66% and 61.33% were observed for OTU and non-OTU species categorization respectively. 36% of sequences were considered as ambiguous under the non-OTU criteria whereas the remaining queries were designated matchless.

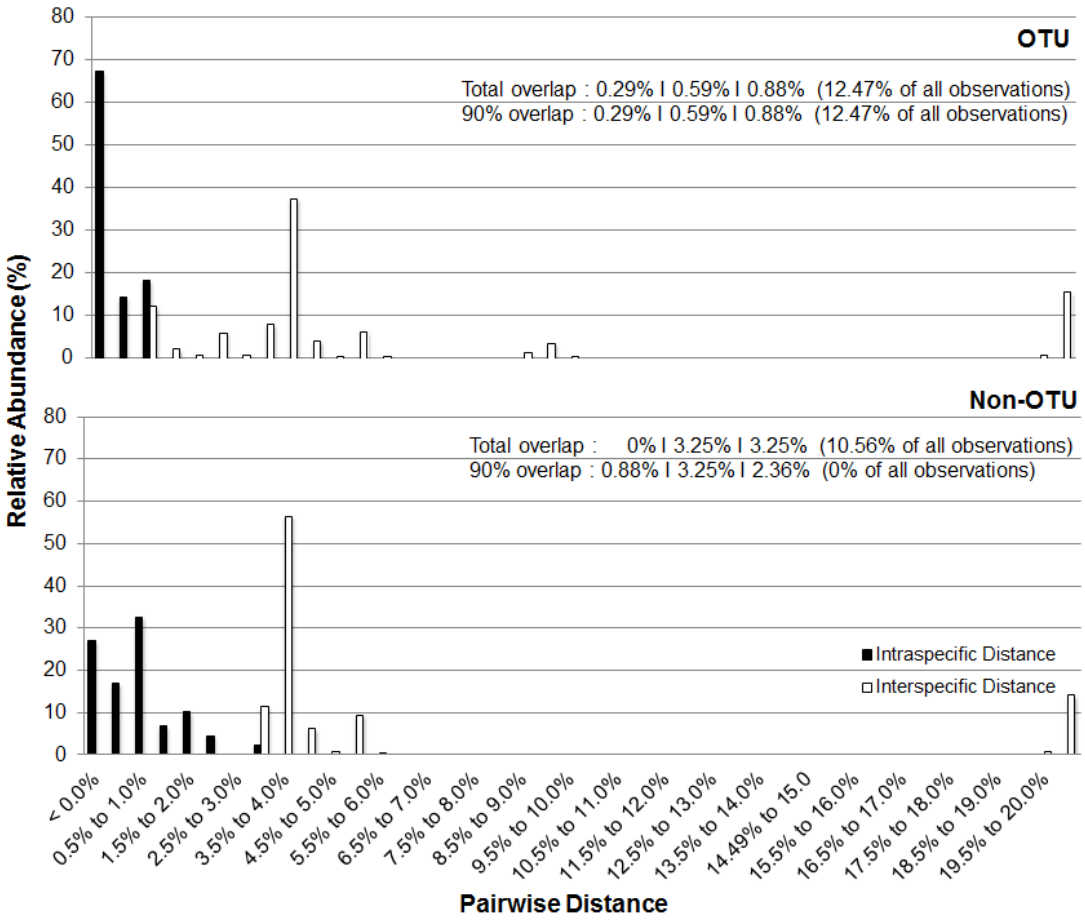

Figure 4.16: Plot of intra- and interspecific genetic distances of the *cox*2-3 spacer with the application of OTU and non-OTU criteria. Numeric values are arranged according to: the difference between the smallest interspecific but intrageneric distance and the largest intraspecific distance | smallest pairwise distance between interspecific but intrageneic sequences | largest pairwise distance between intraspecific sequences; followed by the number of observations affected (in brackets).

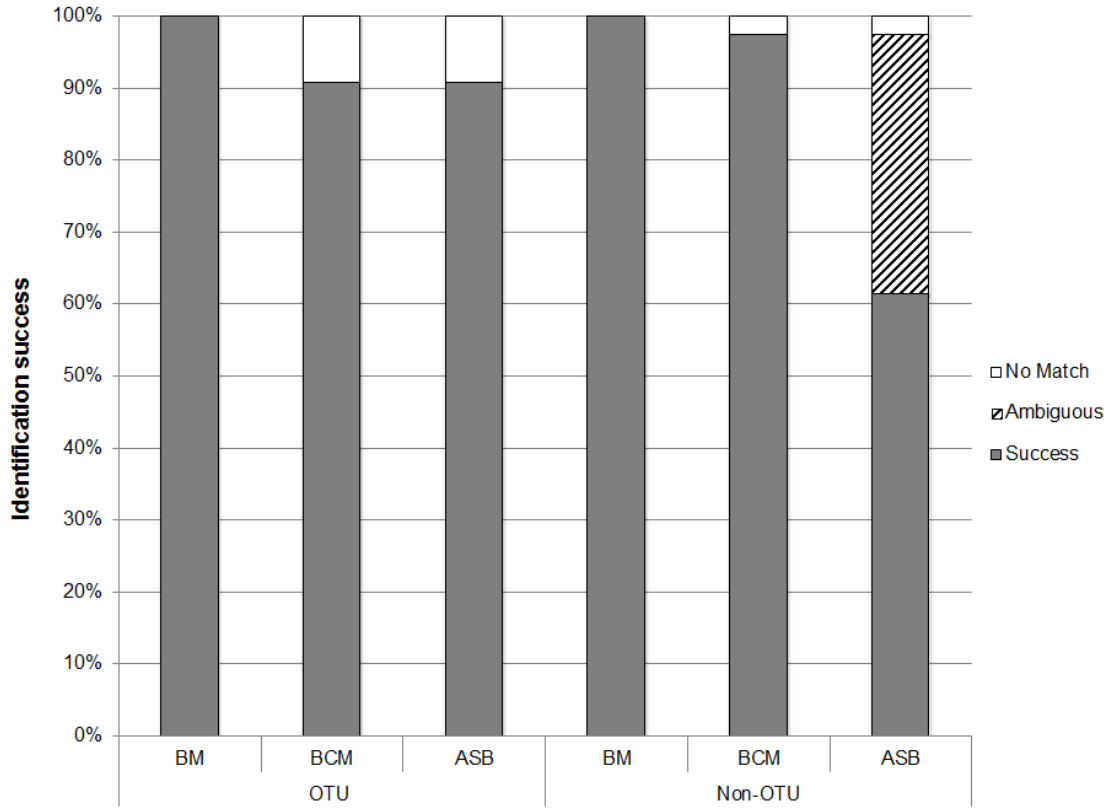

Figure 4.17: Identification success of the *cox*2-3 spacer under Large Dataset Assessment. *Kappaphycus* and *Eucheuma* samples were categorized under either Operational Taxonomic Units (OTU) or non-OTU conditions. Results were generated based on the *Best Match* (BM), *Best Close Match* (BCM) and *All Species Barcodes* (ASB) criteria.

4.4.3.2 Tree-based DNA identification criteria

Similar tree topologies were obtained based on the NJ, ML, MP and BI algorithms for the *cox*2-3 spacer marker and are thus compiled and summarized as Figure 4.18, which is identical to that of Figure 4.8 (excluding the outgroup), albeit the different annotations according to OTU and non-OTU taxonomic assortments. The NJ, MP and BI phylogenetic trees are available as supplementary data (Appendix O-Q).

Tree-based DNA identification was assessed using the NJ tree without taking into account the *Solieria* outgroup not used in the NJ dataset. In accordance to identification criteria by Hebert and co-workers (2003a), 100% identification success is reported when tree-based identification is based on the OTU concept. When taxa are queried using conventional taxonomic naming (non-OTU), percentage of successful

identification is reduced to 94.6%, where the remaining 5.4% indicated misidentifications. Application of Meier et al's (2006) identification criteria returned comparatively lower successful identification rates, where 95.9% success and 4.1% ambiguity was recorded for OTUs; and 67.6% successful identification, 27% ambiguity and 5.4% misidentification for non-OTUs.

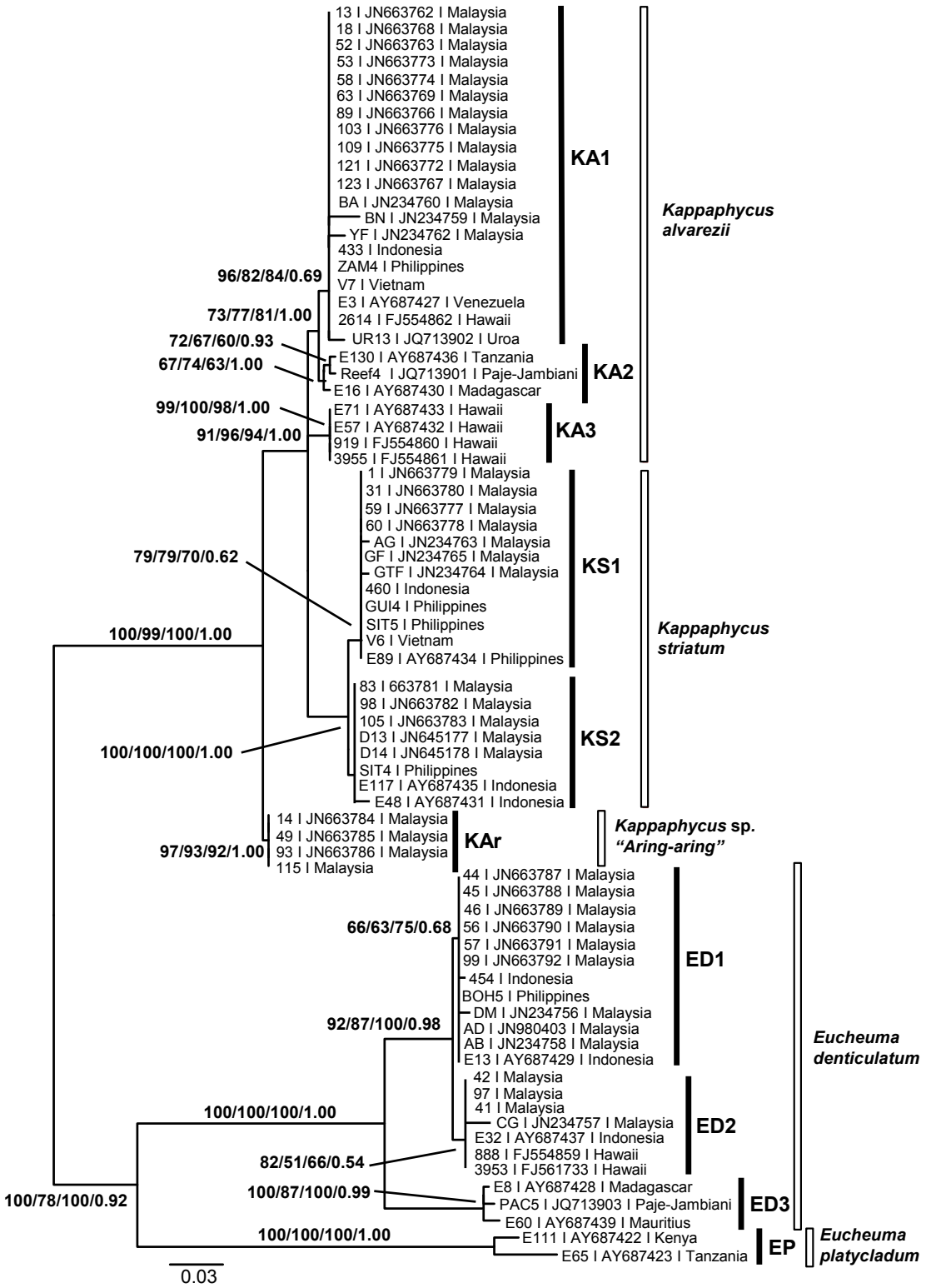

Figure 4.18: Maximum likelihood 50% majority-rule consensus tree based on the *cox*2-3 spacer**.** Number at nodes is arranged according to NJ bootstrap support/ ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. *Large dataset assessment*: black bars indicate Operational Taxonomic Units (OTU), whereas white bars represent non-OTU clusters.

CHAPTER 5: DISCUSSION

5.1 Sample collection and processing

Cultivated specimens of *Kappaphycus* and *Eucheuma* can be easily retrieved from farm monolines. Wild specimens often grow attached to substratum by means of a holdfast, often at intertidal areas. Wild seaweeds are mostly damaged or deformed due to adverse environment pressure or herbivory, thus rendering on-site identification challenging. The occurrence of cystocarpic seaweeds was not consistent for all species: no fertile *K. alvarezii* or *Eucheuma* samples were observed in Malaysia throughout the entire span of the project whereas cystocarpic *K. striatus* and "*Aring-aring*" varieties (supported by molecular data later) were very common. These cystocarpic seaweeds were mostly concentrated around the Sabangkat Island of Sabah, East Malaysia. The rare occurrence of cystocarpic *K. alvarezii* can be ascribed to the limited viable spore production, as pointed out by Russell (1983).

The difficulties in sample processing of *Kappaphycus* and *Eucheuma* are well known (Doty 1988; Doty and Norris 1985). The relatively large sizes of these carrageenophytes proved a challenge in transportation and processing. The gradual release of moisture from the drying thalli (also known as the "sweating" effect) will result in the formation of salt crystals (Potassium Chloride) which served as temporary preservatives for the herbarium when being dried (Neish 2003). Regardless, when pressed and air-dried, the amount of moisture released will still be conducive for fungal growth. Hence, the intervals at which the C-Fold towels are changed must be frequent i.e. on a daily basis for the first two weeks. The usage of newspapers as part of the herbarium press is advisable as they are cheap and easily available on sampling trips, and they absorb moisture better.

Preparation of silica gel specimens for subsequent DNA isolation was straightforward, with extra care needed in making sure that epiphytes, particularly those of the same Division e.g. *Polysiphonia* etc. are properly removed from the excised thallus of the *Kappaphycus* and *Eucheuma* specimens to avoid erroneous results in subsequent steps. Thalli with endophytes should be avoided.

5.2 Morphological observations

Morphological analysis of cultivated *Kappaphycus* and *Eucheuma* (Table 4.2) samples based on plant size, color, branch diameter, branching patterns, and thalli texture was shown to be capable of differentiating five varieties of *K. alvarezii*, two varieties of *K. striatus*, one variety of *Kappaphycus* sp. (*Aring-aring*) and two varieties of *E. denticulatum*; as commonly suggested by local farmers. Cultivated samples, grown under better farm management, are often more robust and healthy in terms of growth. As aforementioned, wild specimens proved difficult to differentiate morphologically mostly due to thallus deformities. Fertile seaweeds also exhibit vast morphological differences compared to cultivated ones. It is therefore difficult to compare morphologies of wild and cultivated specimens, worsened by the fact that "wild" seaweeds may be residuals that drifted from nearby farms.

The identification of cultivated and undamaged *K. alvarezii*, *K. striatus*, *Kappaphycus* sp. "*Aring-aring*", *E. denticulatum* "*Spinosum*" and *E. denticulatum* "*Cacing*" is fairly easy. These seaweeds can be distinguished from one another based on three main identification criteria, namely the branch diameter, branching patterns and the texture of the thalli. Despite the irregular branching patterns, *K. alvarezii* tend to exhibit the largest branch diameters, followed by *K. striatus*, *E. denticulatum* "*Cacing*", *Kappaphycus* sp. "*Aring-aring*" and *E. denticulatum* "*Spinosum*" (Table 4.2). *K. striatus* displayed the highest branching frequency, with branching intervals of less than 2 cm,

attaining up to five degrees of branching, forming a compact, dorsally asymmetrical bunch. *Kappaphycus* sp. "*Aring-aring*" can be differentiated from the others by the overall smaller size and relatively slimmer branch diameters as well as the slim and attenuated branch apices. Unlike the fleshy and cartilaginous nature seen in *Kappaphycus*, the thallus of *E. denticulatum* "*Spinosum*" varieties is brittle and inflexible, with characteristic pinnate or pectinate spines throughout. The morphological features of *Cacing* specimens (shown by molecular results to be also *E. denticulatum* (Ganzon-Fortes et al. 2011)) appeared different from those of *E. denticulatum. Cacing* varieties exhibit a cartilaginous, smooth and non-brittle thallus in which pinnate spines are significantly fewer than in *Spinosum* varieties. These spines are regularly arranged and widely spaced from one another. *Cacing* varieties also exhibit characteristic tiny, determinate and pointed growth protrusions on branch terminals which may be singular to multifurcations. These morphological observations match those on the Philippines "*endong*" variety (Ganzon-Fortes et al. 2011). However, the deviations in terms of morphology do not fulfil certain characteristics coined for *E. denticulatum*- occurrences of spines in whorls at regular intervals and conical spines (Doty 1988; Doty and Norris 1985). This suggests the need to re-examine the taxonomy of the associated genus. Despite exhibiting somewhat "intermediate" morphological characteristics of both *Kappaphycus* and *Eucheuma, Cacing* varieties can still be differentiated from *Kappaphycus* via the regularly arranged and mostly opposite branching, the smooth thallus surface and the short and tiny branchlets or ramuli on branch terminals.

Most of the local varieties of *Kappaphycus* are not supported by molecular data (to be discussed later) despite the difference in terms of color and putative external morphologies. The variation in terms of color and morphological characteristics may be attributed to the interaction between light, water currents, water depth as well as nutrient availability (Góes and Reis 2011; Munoz et al. 2004; Santelices 1999; Thirumaran and

Anantharaman 2009). For instance, *K. alvarezii* domesticated around the Pangkor Islands (Peninsular Malaysia) are generally more robust under the relatively nutrient rich and strong currents in the area, but are in turn more exposed to severe grazing, seasonal epiphytes, and sediment problems. All these factors contribute to morphologies different than those usually observed under proper farm management. Morphological studies indicate a marked difference between cultivated and wild *K. striatus*. Phylogenetic analyses of *K. striatus* samples (to be discussed later) revealed two distinct, monophyletic clades B1/KS1 (mostly cultivated) and B2/KS2 (mostly wild) which suggest the existence of only two distinct varieties or genotypes. Further examination of more samples based on both morphological and molecular analyses is needed to verify this finding. The cultivated *Aring-aring* varieties (Table 4.2 and Figure 4.2G-I), often with shades of pale brown or green, are slim with long, slender, and pointed branch apices. Morphological observations has also shown that the hemispheric and swollen cystocarps with dark, central patches of carposporangia exhibited by wild *Kappaphycus* sp. (*Aring-aring*) (Figure 4.2I) is different from the cone-shaped and somewhat pointed cystocarps of wild *K. striatus* samples (83, 98, and 105) (Figure 4.2C-F) of this study (fertile *K. alvarezii* was not observed). However, it is uncertain whether the pointed cystocarps observed in the *K. striatus* samples were due to immaturity. Morphological observations (Doty and Norris 1985) and molecular data both confirmed the Malaysian *Spinosum* variety (Figure 4.3A, B, D and E) as being *E. denticulatum*.

Wild, tiny or deformed specimens were virtually impossible to identify accurately based solely on morphology and thus require supplementary data using genetic markers. Similarly, to date it is still difficult to differentiate between seaweeds of the tetrasporophytic and the gametophytic (yet non-fertile) life stages on-site. Nevertheless, ploidy determination is possible through the application of confocal microscopy (Zitta et al. 2011). Fertile specimens will undoubtedly provide insights or

perhaps distinctive morphological criteria for a better taxonomic understanding of both *Kappaphycus* and *Eucheuma*. Efforts are underway to find *K. alvarezii* specimens around the Karindingan Island (type locality), and also fertile seaweaeds of each species of *Kappaphycus* and *Eucheuma* that are available in the seas of Malaysia.

It is worth noting here that the drying and herbarium pressing of *Kappaphycus* and *Eucheuma* results in severe loss of structural integrity and morphological characteristics (Doty 1988; Doty and Norris 1985; Neish 2003), thus making direct morphological comparisons with type herbarium specimens taxing and somewhat pointless. Photographs of fresh specimens are thus of equal importance for future references.

5.3 DNA extraction and amplification

Upon drying, the thalli of (especially) *Kappaphycus* and *Eucheuma* tend to harden, which pose difficulties for the grinding step, even when liquid nitrogen is used. It is therefore advisable to only choose terminal branches for silica gel preservations whenever possible. Samples used for grinding must be completely dried since these carrageenophytes have good water retention capabilities, to prevent ice formation when exposed to liquid nitrogen. Additionally, the hardened, dried thalli will have the tendency to spill when ground with "boiling" liquid nitrogen. This can be easily avoided by pressing the ground samples to the base of the 1.5ml tube using a micropestle until most of the liquid nitrogen has evaporated.

The amount of starting materials used for DNA extraction is important as unlike normal animal or plant tissues, *Kappaphycus* and *Eucheuma* are rich in carrageenan, which will form gels and thus affect proper DNA isolation. Additionally, after the lysis buffer is added and the sample incubated at 65˚C, constant vortexing at intervals is required to prevent gel formation, apart from promoting homogenization.

Longer incubation durations can be applied in order to completely dissolve the carrageenan for better mixing.

For PCR amplification of samples, the amount of starting materials were, after optimized, determined to be most suitable at DNA concentrations of ~25ng (measured using Nanodrop 2000, Thermo Scientific, USA). Too much starting DNA will occasionally result in unsuccessful amplifications, regardless of the molecular marker used. Although the exact reason why this occurs is not yet known, it might possibly be due to the presence of polysaccharides or other substances (eluted during DNA isolation) that interfered with the normal functioning mechanism of the PCR components. DNA smearing was sometimes observed as well when starting DNA concentrations were too high, resulting in non- specific amplifications. Optimized amounts of starting DNA will produce sufficient amounts of amplicons (per 20µl reaction volumes) for direct purification with only one PCR run.

5.4 Molecular Phylogenetics and DNA Barcoding of *Kappaphycus* and *Eucheuma*

5.4.1 Molecular marker assessments and potential DNA barcodes

Although all five genetic markers (*cox*1, *cox*2, *cox*2-3 spacer, *rbc*L and RuBisCO spacer) used in this study were capable of phylogenetically reconstructing the systematics of *Kappaphycus* and *Eucheuma*, the extent of each marker's resolution is different, thus requiring individual assessments. The RuBisCO spacer was earlier reported to be relatively poorer in resolving the phylogeny of these red algae (Conklin et al. 2009; Zuccarello et al. 2006), which was also supported by the findings of the present study; and it was thus omitted from subsequent assessments.

Potential DNA barcodes which are essentially genetic markers need to satisfy certain prerequisites prior to recommendation for large-scale construction of DNA barcode libraries. The criteria are: short length (<1.5kb), universality, ease of amplification, preferably protein coding (ease of alignment), good resolution power and of course accurate species identification (Jinbo et al. 2011). These criteria apply to the genetic markers used for molecular phylogeny (also an application of DNA barcoding) as well, and are thus discussed altogether within this context.

The dataset in the present study represents a small conglomerate of selected and commonly available *Kappaphycus* and *Eucheuma* samples from Southeast Asia. Sampling size is restricted to an amount supposedly cost-effective for molecular marker assessments as potential DNA barcodes. Owing to the relatively scarce records of *Kappaphycus* and *Eucheuma* in the GenBank, this study will also serve as a preliminary work to increase the amount of reference data using the potential DNA barcode, which can eventually be established as a barcode library.

The accuracy of a DNA barcode is largely determined by the magnitude of overlap between intraspecific variation and interspecific divergence. Ideally, the

absence of an overlap would render species identification straightforward but this condition is virtually non-existent in very large datasets, in which the less the overlap, the more accurate it is for species identification (Hebert et al. 2004; Jinbo et al. 2011; Meyer and Paulay 2005). Results indicated that no overlaps were observed for the *cox*1, *cox*2, *cox*2-3 spacer and the *rbc*L genetic markers and have minimal impact on the accuracy of species identification for *Kappaphycus* and *Eucheuma* in this study (Figure 4.13). However, the absence of overlaps may be attributed to the small number of taxa in Southeast Asia (particularly so the genus *Eucheuma*) at this time, and is expected to change as more samples of different species or from different geographical locations are included in the future (Jinbo et al. 2011; Meier et al. 2008). The presence of a "Barcoding Gap" (the absence of overlaps between intra- and interspecific genetic variations) may also be explained by the relatively lower intraspecific genetic diversity as compared to those reported in arthropods (Hebert et al. 2003a, Meier et al. 2006; Meyer and Paulay 2005; Wiemers and Fiedler 2007)

All four molecular markers collectively showed an identification success of 100% for the BM criteria (Figure 4.14). Under the BCM criteria, the plastid encoded *rbc*L gene exhibited the highest identification success of 100% whereas the mitochondrial encoded *cox*2, *cox*2-3 spacer and *cox*1 spacer recorded slightly lower success of 96.6%, 93.1% and 79.3% respectively (Figure 4.14). Inaccurate identification of species was not reported for all four molecular markers. The eventual increase in conspecific DNA sequences for *Kappaphycus* and *Eucheuma* is expected to reduce the probability of queries not meeting any matching sequences. All these results reflect the relatively less variable *rbc*L region as compared to mitochondrial counterparts, which was also reported by Geraldino et al. (2006) and Yang et al. (2007). Although widely championed as a good potential DNA barcode, the relatively higher intraspecific variation of the *cox*1 marker (up to 0.43% genetic distance for *Kappaphycus*; up to 0.07%

for *Eucheuma*) requires caution in avoiding misidentifications. Similar or higher intraspecific patterns were pointed out in previous studies of rhodophytes (Geraldino et al. 2006; Geraldino et al. 2009; Robba et al. 2006; Saunders 2005; Wiriyadamrikul et al. 2010; Yang et al. 2007)

The tree-based DNA identification approach (Figure 4.15) returned 100% success in species identification for all four molecular markers using the criteria by Hebert and co-workers (2003a). Although the application of the relatively stricter identification criteria by Meier and co-workers (Meier et al. 2006) generally lowered the successful identification scores, they were still higher compared to the results derived from the distance-based approach. For instance, *cox*1 and *cox*2 showed a higher identification success of 89.7% and 96.6% respectively (Figure 4.15A-B). Ambiguous identification in this study was mostly caused by queries that formed a sister group to a cluster of conspecific sequences. This is also expected to decrease as more reference sequences are deposited in the GenBank for *Kappaphycus* and *Eucheuma*. Contrary to the better results obtained by the tree-based method in this study, empirical studies involving much larger taxa coverage have reported the preference of distance-based assessment over tree-based ones in terms of accuracy and robustness (Meier et al. 2006; Virgilio et al. 2010). The accuracy and resolving power of a genetic marker for phylogenetic inferences is especially evident under the tree-based DNA identification criteria, of which hypothesis and solid conclusions are based. An ideal molecular marker for molecular systematics should display sufficient genetic variability for proper phylogenetic delineation of *Kappaphycus* and *Eucheuma* samples.

Based on both the distance and tree-based DNA identification approaches, the absence of overlaps between inter- and intraspecific genetic variability of the *rbc*L gene (Figure 4.13) as well as its relatively conserved nature (Figure 4.15D) serve as barcode criteria for *Kappaphycus* and *Eucheuma*. However, the reduced genetic variation would

also imply the incapability of the *rbc*L marker to detect incipient speciation or genetic diversity within species (Hollingsworth et al. 2011; van Velzen R. et al. 2012). This will not only result in an underestimate of the actual genetic richness of these seaweeds, but will most likely overestimate interspecific variation as well due to the unavailability of closely related species (Jinbo et al. 2011), thereby affecting the accuracy of phylogenetic inferences. The drawbacks of the plastid encoded *rbc*L marker can be accounted for using supplementary molecular data generated from the relatively more variable, mitochondrial derived *cox*1, *cox*2 or *cox*2-3 spacer. The concept of combined molecular data, in this case of molecular markers, is not new considering the occasional failures of the DNA barcodes in correctly and consistently identifying species, as observed in Karner blue butterflies (Gompert et al. 2006), seagrasses (DeSalle et al. 2012) and flowering plants (Kress 2005).

Utilization of genomic DNA from different origin i.e. mitochondrial, nuclear or plastid with different evolutionary rates would offer a better picture with respect to gene genealogy and evolutionary lineage. However, nuclear-derived genetic markers i.e. 18S SSU and 28S LSU were dismissed in this study following reports of their low genetic variability (Conklin et al. 2009; Geraldino et al. 2006; Le Gall and Saunders 2010; Olson et al. 2004; Sherwood et al. 2010) which does not satisfy the criteria of a good DNA barcode, at least not when compared to plastid or mitochondrial markers. In terms of plastid markers, DNA barcoding of rhodophytes usually involves the UPA and *rbc*L. Although the UPA barcode was reported to be less accurate than *rbc*L for green macroalgae due to its lower species resolution and higher contamination rates (Saunders and Kucera 2010), a recent survey on the Hawaiian freshwater rhodophytes has shown that both markers generated similar phylogenetic and phylogeographic patterns for certain taxa (Carlile and Sherwood 2013). However, the *rbc*L barcode still offers wider taxon coverage, especially since it is officially accepted as a DNA barcode for land

plants (Consortium for the Barcode of Life 2009; Hollingsworth et al. 2009; Hollingsworth et al. 2011; Jinbo et al. 2011; Newmaster et al. 2006). Mitochondrial-encoded genetic markers are often associated with easy amplifications, good genetic variations and a fixed pattern of maternal inheritance- good attributes for DNA barcoding. Upon the initial introduction by Herbert and colleagues (2003), the application of the *cox*1 marker quickly extended to the rhodophyte taxa, with promising results and prospects (Robba et al. 2006; Saunders 2005). This marker has since then been applied for average to large-scale DNA barcoding of rhodophytes (Freshwater et al. 2010; Geraldino et al. 2009; Kim et al. 2010; Saunders 2008; Sherwood et al. 2010; Wiriyadamrikul et al. 2010; Yang et al. 2007), improving the taxonomic classifications of Rhodophyta. Still, even these markers suffer from setbacks in terms of universality, with instances where it does not work well or at all for certain taxa such as the fungi, plants and certain groups of algae (Chase and Fay 2009; Jinbo et al. 2011).

The *cox*1 and *cox*2 DNA markers hold better potential as DNA barcodes (or markers essentially for phylogeny) compared to the *cox*2-3 spacer because of their protein coding properties (Chase et al. 2007; Jinbo et al. 2011; Robert et al. 2011). These markers are more conserved across taxa with less indel mutations and are much simpler to check for errors through amino-acid translation. Lack of recombination and uniparental inheritance of mitochondrial markers are added advantages (Zuccarello et al. 1999). The relatively high mitochondrial copy numbers also enable ease of amplification. Based on our dataset, the *cox*2 genetic marker, with its moderately high interspecific divergences within generic level and non-existent intraspecific variation, has demonstrated relatively successful results in terms of species identification in this study and is thus regarded as a potential mitochondrial DNA barcode for *Kappaphycus* and *Eucheuma*. Large scale application of this molecular marker is expected to bring about advancements mainly in the field of agriculture, taxonomy and biomonitoring.

However, considering the already widely utilized *cox*2-3 spacer exhibited almost similar barcoding traits as that of the mitochondrial *cox*2 marker, continual implementation is recommended, in spite of its non-coding nature. The implementation of the *cox*2 molecular marker across taxa may pose a problem as well since the full potential of the *cox*2 marker has yet to be tested in other rhodophytes. Additionally, the massively abundant and readily available *cox*1 sequences within the GenBank (although not extensively tested on *Kappaphycus* and *Eucheuma* at that time, *cox*1 has been proposed as the potential universal DNA barcode for red algae) is apparently more practical and economical to work with, despite the relatively higher intraspecific variations which may reduce the accuracy for species identification (Freshwater et al. 2010; Geraldino et al. 2006; Geraldino et al. 2009; Robba et al. 2006; Saunders 2005; Wiriyadamrikul et al. 2010; Yang et al. 2007). Based on the results of the present study, the authors have come to a consensus that the mitochondrial *cox*2-3 spacer was overall the best potential DNA barcode for *Kappaphycus* and *Eucheuma*, whereas the combined mitochondrial-encoded *cox*1 and the plastid-encoded *rbc*L markers serve better as DNA barcodes encompassing the entire rhodophyte taxa.

The robustness and efficiency of DNA barcoding tends to increase with increased reference sequences and taxonomic scrutiny (Hebert et al. 2003a; Jinbo et al. 2011; Meyer and Paulay 2005; Virgilio et al. 2010). This includes genetically distinct individuals within a species' range to account for molecular markers with high variability such as the *cox*1 (Meier et al. 2006). Although this would greatly increase GenBank data and hence lead to an inevitable increment in terms of computational demand, larger and properly annotated datasets would expedite phylogeography, evolutionary biology, and biodiversity or population genetic studies in the future.

5.4.2 Large dataset assessment

The Large Dataset Assessment was carried out to determine the effects of larger, empirical datasets on the effectiveness of a potential DNA barcode. The *cox*2-3 spacer was chosen for this assessment as it is currently the most widely used genetic marker, with the most GenBank entries for *Kappaphycus* and *Eucheuma* (Figure 4.18). Several specimens of unknown identity or unreliable identification i.e. *Kappaphycus cottonii*, *Eucheuma isiforme* etc. were excluded as specimens that are wrongly identified will affect the accuracy of a DNA barcode system. Morphological plasticity of these seaweeds has rendered species identification and description challenging, even to seasoned taxonomists. As of now, distinctive morphological characters are still undiscovered despite ample DNA evidence supporting the possible existence of new, or perhaps cryptic species. This has led to the amplification of Operational Taxonomic Units (OTU) in this context; representing genotypic diversity possibly overlooked via conventional morphological traits.

Distance-based results on the *cox*2-3 spacer dataset have shown that the incorporation of more sequences decreases the ‘‘Barcoding Gap’’ (when not overlapping), and to the extent of forming overlaps between inter- and intraspecific divergence. When *Kappaphycus* and *Eucheuma* species were regarded as non-OTUs, the ‘‘Barcoding Gap’’ for the entire length of *cox*2-3 spacer genetic marker decreased from 0.27% to 0% (Figure 4.16). When under OTU assortment, the intra- and interspecific genetic divergences formed an overlap of 0.29%. These observations were not surprising considering the larger sample size would eventually lead to higher occurrence of specimens with varying genetic composition (Jinbo et al. 2011; Meier et al. 2008). This would undoubtedly affect the identification accuracy of DNA barcoding. BCM results saw a slight dip from 93.1% to 90.7% when samples were regarded as OTUs (Figure 4.17). Similar patterns were observed for tree-based DNA identification

under Meier's (2006) criteria, where identification success reduced from 96.6% to 95.9% for non-OTUs, caused mainly by ambiguous sequences. These ''singleton'' sequences or query sequences sister to known species can be avoided with increased data and taxonomic rectification or reformation.

The distance-based All Species Barcodes (ASB) assessment (Figure 4.17), being relatively stricter compared to BM and BCM, has reflected our poor taxonomic comprehension on *Kappaphycus* and *Eucheuma*, in which at our current state (represented by non-OTU species assortment); a mere 61.3% of queries could be identified correctly using DNA barcoding. This figure is probably overestimated considering the large amount of *Kappaphycus* and *Eucheuma* yet to be discovered and described. Misidentifications under non-OTU conditions were caused by the African (KA2) and Hawaiian (KA3) *K. alvarezii* specimens, where the latter was poorly resolved using the *cox*2-3 spacer and *cox*1 (appendix R) molecular markers. Still, combined *cox*2-3 spacer and RuBisCO spacer data has shown with moderate support that the KA3 specimens were more closely related to *K. alvarezii* than *K. striatus* (Zuccarello et al. 2006). This implies the possible limitation in resolving power of individual markers at intraspecific levels although the *rbc*L and *cox*2 molecular markers are yet to be tested. On the other hand, ASB has returned relatively higher identification successes (90.7%) when *Kappaphycus* and *Eucheuma* species were regarded as OTUs, thus providing invaluable insights DNA Barcoding of *Kappaphycus* and *Eucheuma.*

5.4.3 Molecular phylogenetics and haplotype networks

The *cox*2-3 spacer and the RuBisCO spacer genetic markers offered the widest phylogenetic coverage of *Kappaphycus* and *Eucheuma* specimens due to the large numbers of GenBank reference sequences. Concatenation of both the *cox*2-3 spacer and RuBisCO spacer datasets were already reported by Zuccarello and co-workers (2006), in which the taxonomic position of the Hawaiian *K. alvarezii* (Clade A3) does not conform to that derived based on the RuBisCO spacer or concatenated *cox*1-*cox*2-3 spacer dataset of this study (Appendix R). As a result of this data discrepancy, possibly due to lack of additional sequences from other genetic markers for comparison or inadequate marker resolution, the concatenation of the *cox*2-3 spacer and RuBisCO markers was not pursued in this study.

The *cox*1, *cox*2 and *rbc*L datasets, despite being relatively limited in terms of taxa, offered decent phylogenetic resolution to these commercially important seaweeds. Apart from the RuBisCO spacer, all molecular markers offered more or less similar tree topologies which will be discussed altogether.

5.4.3.1 *Kappaphycus*

Based on molecular results (Figure 4.6-4.9), *Kappaphycus alvarezii* (Clade A1) appeared to be the most commonly cultivated genotype, and was reported throughout Southeast Asia, Africa, Columbia, Panama and Hawaii. *cox*2-3 spacer genealogy suggested that the African and Hawaiian strains of *K. alvarezii* A1, among others, may possibly be introduced strains, presumably traceable back to the Philippines. This is not surprising as there were efforts to introduce foreign, good strains of *Kappaphycus* for cultivation in the past (Conklin et al. 2009; Pickering et al. 2007; Zuccarello et al. 2006). Members of this clade are probably synonymous to the type *K. alvarezii* (*E. alvarezii* then) described by Doty (1985). All cultivated *K. alvarezii* collected throughout

Southeast Asia in this study reside within Clade A1, indicating that most of the local varieties, at least those in Malaysia, were not genetically dissimilar despite the variations in terms of morphology and color. For instance, the *Tambalang*, *Tangan-tangan* (Loving Beauty) and *Buaya* (Crocodile) varieties, are conspecific, regardless of their color ranges. Although *K. alvarezii* A1 can be found cultivated in the farms within Africa and Hawaii, these two regions have their own exclusive genetic strains of *K. alvarezii*, respectively grouped in Clade A2 and A3. The *K. alvarezii* from Madagascar and Tanzania collectively formed a monophyletic clade inferred to be sister to the *K. alvarezii* of Clade A1, indicating a significant genetic difference between these two genotypes. Although not covered in this particular dissertation, future work should emphasize in better morphological scrutiny of these seaweeds, which might end up as a different species as compared to the originally described *K. alvarezii*. Similar observations were observed for a large amount of Hawaiian "*K. alvarezii*" specimens, which are clustered together with high support (>90% or 0.90 for ML, MP and BI for *cox*1 and *cox*2-3 spacer). However, the taxonomic standing of these samples (Clade A3) is uncertain based on the individual resolution of each genetic marker, where Clade A3 was inferred as polytomy to both *K. alvarezii* and *K. striatus*. Zuccarello and co-workers (2006) showed that members of the Clade A3 were more closely related to *K. alvarezii* than *K. striatus* using a combination of both the *cox*2-3 spacer and RuBisCO spacer genetic marker. However, the dataset used in this study, albeit having merely one Hawaiian sample with sequences for both the mitochondrial *cox*1 and *cox*2-3 spacer DNA markers; appeared to disagree with the findings by Zuccarello. A larger amount of sequences, preferably of *cox*1, *cox*2-3 spacer and *rbc*L would be required in order to effectively determine the taxonomic position of these "*K. alvarezii*" from Hawaii. Of course, the morphology of these carrageenophytes should be looked into as well, serving as primary data for better taxonomic differentiation. The occurrences of the

African and Hawaiian-specific genetic strains of "*K. alvarezii*" indicate that not all of the *K. alvarezii* used for commercial cultivation in these areas originated from the Philippines. Wild specimens of *K. alvarezii* were uncommon, let alone fertile ones. Increased efforts should be carried out in order to locate wild or native *K. alvarezii* within Southeast Asia, so that the natural distributions of this particular species of seaweed can be archived, and if needed, monitored and maintained. Sampling efforts should also be concentrated on the type locality for *K. alvarezii*, which is near the Karindingan Island of Sabah, Malaysia.

Although the initiation of *Kappaphycus striatus* cultivation was not really documented, the relatively rapid growth and compact nature had rendered *K. striatus* a formidable alternative to *K. alvarezii* cultivars. *K. striatus* specimens constitute a monophyletic Clade B with strong support (>80% or 0.80 for ML, MP and BI for *cox*2, *cox*2-3 spacer and *rbc*L). Although no apparent morphological differences were observed, *K. striatus* specimens were clustered into two different genotypes, namely Clade B1 (*K. striatus* B1/KS1) and B2 (*K. striatus* B2/KS2). Apart from a wild *K. striatus* "*Cottonii*" GUI4 from the Philippines, all members within Clade B1 were cultivated specimens. On the other hand, apart from a cultivated Philippine *K. striatus* "*Kab-Kab Green*" SIT4, members of Clade B2 were all collected from the wild. The paucity of morphological differences in between members of Clade B1 and B2 suggest that members of Clade B2 might be a cryptic species. There is a clear distinction between Malaysian and Indonesian *K. striatus* samples of Clade B1 and B2, where the former is composed of mainly cultivated species, whereas the latter comprises of wild specimens. Emphasis can be placed on domesticating these wild *K. striatus* specimens which may have potentially better growth and carrageenan yields. Wild and fertile *K. striatus* seaweeds showed no differences in genetic affinity to the *K. striatus* cultivars,

which is not surprising since genetic compositions are not expected to change with alternate of generations.

The local "*Aring-aring*" variety in Malaysia was earlier shown to be morphologically different from *K. alvarezii* and *K. striatus* counterparts. In terms of molecular results, all "*Aring-aring*" samples and a wild specimen 93 were resolved with monophyly as Clade C with moderate to high support (>75% or 0.75 for ML, MP and BI for *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L) (Figure 4.6-4.9). Clade C occurs as a sister taxa to the clade composed of *K. alvarezii* and *K. striatus* (Clade A and B). This indicates that the "*Aring-aring*" variety is genetically distant from both *K. alvarezii* and *K. striatus* but shared a common ancestor and a particular point in time; and since these seaweeds do not cluster along with *Eucheuma* specimens, the local "*Aring-aring*" variety is considered as a member of the genus *Kappaphycus*, henceforth termed as *Kappaphycus* sp. "*Aring-aring*", which is highly likely a new species. The taxonomic placing of the "*Aring-aring*" variety within *Kappaphycus* is also supported by the fact that local this particular variety produces *kappa*-carrageenan. Although the name "*Aring-aring*" has been used in earlier publications from the Philippines (Villanueva et al. 2011), there were no indications as to whether the literature was referring to the same *Kappaphycus* sp. "*Aring-aring*". However, considering the relatively large amount of wild *Kappaphycus* sp. "*Aring-aring*" specimens (most of which were fertile) observed around the Sabangkat island, it is possible that this species would be available in the Philippines and also along the Makassar Strait due to the sharing of a somewhat similar ecological niche. Future work should include the establishment of *Kappaphycus* sp. "*Aring-aring*" as a new species of the genus *Kappaphycus*, which might be potentially useful as an alternative cultivar to *K. alvarezii* and *K. striatus* considering the relatively good gel strengths (Phang et al. 2010).

The taxonomic status of *Kappaphycus cottonii* was not resolved in this study, which can be attributed to the small amounts of samples collected, in addition to the resolution limits of each genetic marker assessed. The drawback of the RuBisCO spacer in phylogenetic reconstruction is noted and will not be discussed in this context. Based on the *cox*1 (Figure 4.6) and *cox*2-3 spacer (Figure 4.8) genetic markers, the lone *K. cottonii* sample from the Philippines was resolved as a sister taxa to all the aforementioned *Kappaphycus* species, albeit with merely low to no support. Although certain cultivars were named *K. cottonii* or the trade name "*Cottonii*", molecular results have proven that there were no genetic affiliations to the true *K. cottonii*. To date it is believed that no *K. cottonii* has been domesticated for commercialization, and can possibly only be found as wild populations. Due to its rareness, this particular carrageenophyte is very poorly studied. Similar to that of *Kappaphycus* sp. "*Aring-aring*", the *K. cottonii* could potentially be a suitable as a commercial cultivar for carrageenan as well, hence an aspect worth looking into in the future.

No *Kappaphycus procrusteanus* samples were collected in this study although it is listed as a valid species within Algaebase. Although the type specimen was collected from the Sulu Sea of the Philippines, no genetic records are available within GenBank for comparisons; and it is likely that *K. procrusteanus* is not available in Malaysian waters. Sequencing the DNA of the type specimen would offer some insights as to whether this particular species is genetically valid. If valid, increased efforts should be put in to find the natural population and distribution of this *Kappaphycus*, followed by domestication and research.

No matured, male specimens of members of the genus *Kappaphycus*, be it those collected from monolines or those sampled from the wild, were observed throughout the entire span of this study. No tetraspore-producing seaweeds were observed for any cultivated *Kappaphycus* in Malaysia, as opposed to those observed in Brazil (Bulboa et

al. 2008). To date, the environmental conditions deemed necessary for tetraspore and carpospores generation are still unknown. Better monitoring of conditions leading to spore release of *Kappaphycus* would be of great benefits, especially in introducing genetically variable progenies to replace the current cultivars that have been vegetatively propagated for almost four decades. The ability to induce spore release would also offer better understanding on the tetraspore and carpospore dispersal mechanisms, in addition to life cycle studies.

5.4.3.2 *Eucheuma*

Eucheumatoids are relatively poorly studied compared to *Kappaphycus*, possibly due to identification difficulties as well as lower economic value. Samples of *Eucheuma denticulatum*, being the more popularly cultivated species, were clustered into three genotypically distinct subclades. Subclade ED1 represents specimens from Southeast Asia and Hawaii, subclade ED2 from Southeast Asia, Hawaii and Tanzania (Zuccarello G. C. and West J. A. 2006), whereas samples within ED3 were exclusively from Africa. Despite coexisting in the South China and Celebes seas, Southeast Asian *Eucheuma denticulatum* ED1 and ED2 clades do not share similar morphological characteristics. This was shown by Ganzon-Fortes and co-workers (2011), demonstrating the differences between the "*Endong*/*Spaghetti*" variety (ED2) from that of the usual *Spinosum* variety (ED1) of *Eucheuma denticulatum*. The "*Endong*" variety, thence named *E. denticulatum* (Burman) Collins & Hervey var. *endong* Trono & Ganzon-Fortes exhibited smooth, slender terete axes with whorls of determinate branchlets at predictable intervals (Ganzon-Fortes et al. 2011). Results have also revealed that the local *Eucheuma denticulatum* "*Cacing"* variety fits the morphological and biochemical descriptions of the "*Endong*" variety, genetically supported by the *cox*1 and *rbc*L molecular data. The apparent and distinctive morphological characters of the "*Endong*" variety does not fit the original descriptions for *E. denticulatum*, thus suggesting that it

may be a new species instead of a rare variety. With reference to Figure 4.15, this was shown to be potentially true using the near full length *cox*1 genetic marker, where the monophyly of clades ED1 and ED2 were highly supported. Relatively lower nodal supports were displayed by the *cox*2-3 spacer and *cox*2 DNA markers, followed lastly by the *rbc*L marker which fails to clearly indicate monophyly of ED1 and ED2. These patterns are reflective of the genetic variability of each molecular marker and suggest that clades ED1 and ED2 are probably undergoing divergence or have recently diverged. *E. denticulatum* ED3 was inferred to share a common ancestry with *E. denticulatum* ED1 and ED2, and is to date only reported in Africa. Considering the significantly different morphologies reported for ED2, it would be interesting to relook into the detailed anatomy of the African *E. denticulatum*.

The encounter of only E. denticulatum in this study was unexpected, considering the fact that many *Eucheuma* species were reported throughout Southeast Asia by earlier studies i.e. *E. arnoldii* (Atmadja and Prud'homme van Reine 2012; Nguyen and Huynh 1995; Silva et al. 1996; Silva et al. 1987), *E. crustiforme* (Atmadja and Prud'homme van Reine 2012; Doty 1988; Silva et al. 1987; Weber-van Bosse 1928), *E. isiforme* (Silva et al. 1987), *E. serra* (Atmadja and Prud'homme van Reine 2012; Doty 1988; Guiry and Guiry 2013; Silva et al. 1996; Silva et al. 1987). This phenomenon is probably a result of insufficient sampling coverage of wild seaweed populations around Sabah, especially those in deeper waters. Increased efforts in terms of sampling would be required in order to collect and genetically document these Eucheumatoids, which are urgently needed to elucidate the systematics of *Eucheuma*.

5.4.3.3 Haplotype networks of *Kappaphycus* and *Eucheuma*

Haplotype analyses identify specifically down to each nucleotide differences between samples, thus establishing a picture of the gene genealogies of a particular population. Although the most common *Kappaphycus alvarezii* cultivars were grouped together within Clade A1 by the respective *cox*1, *cox*2, *cox*2-3 spacer, *rbc*L and RuBisCO spacer phylogenetic trees, haplotype analyses based on the most commonly used *cox*2-3 spacer (Figure 4.11) has shown a more specific of haplotype links among these specimens. Three other haplotypes were observed based on the results, namely UR13 (Halling et al. 2012), BN and YF. Although GenBank entries BN and YF are unpublished, the results indicate the possibility of genetic variations for the main commercial *K. alvarezii* cultivars. No *Kappaphycus* specimens collected in the present study shared similar haplotypes as those unique to Hawaii (57/71A) or Africa (16, 130, Reef4). Higher genetic diversities were observed for *K. striatus* compared to first reported by Zuccarello et al. (2006). This includes new haplotypes D14, AG and GTF, from Malaysia. Haplotype D14 fits into the hypothetical ancestral haplotype, which is one nucleotide closer to *K. alvarezii* haplotype 3 than haplotype 117 is, indicating a possible, more ancestral genotype. Remaining *K. striatus* samples were grouped within the more common haplotypes 89 and 117. *Kappaphycus* sp. "*Aring-aring*" showed 14 bp difference from *K. alvarezii* haplotype 3, and 22 base pair differences from *K. striatus* haplotype 117, indicating its significant distinction in terms of genotypic composition.

The haplotype connections of *Eucheuma denticulatum* specimens (Figure 4.12) were generally straightforward, with no genetic affinities to African haplotypes, which are at least 30 bp different from those occurring in Southeast Asia. The two most common haplotypes within the waters of Southeast Asia are haplotypes 13 and 32, corresponding respectively to *E. denticulatum* (Burman) Collins & Hervey (as *E.*

spinosum) and *E. denticulatum* (Burman) Collins & Hervey var. *endong* Trono & Ganzon-Fortes (Ganzon-Fortes et al. 2011). Local "*Spinosum*" varieties were clustered within haplotype 13, whereas "*Cacing*" varieties were grouped within haplotype 32, which are 3 bp different from one another. Three new haplotypes 454, CG and DM were established. The Indonesian sample 454 showed "intermediate" genetic compositions between haplotypes 13 and 32, where the genotypic variations may be linked to geographical differences. GenBank entries CG and DM occur as different genetic varieties than the usual haplotypes 13 and 32.

The monitoring and genetic archiving of haplotype networks for *Kappaphycus* and *Eucheuma* will enable researches to keep track and identify potential, genetically superior strains for commercial cultivation in the future. This is important considering the loss of genetic vigor of the same cultivars that have been vegetatively propagated for almost four decades (Conklin et al. 2009; Halling et al. 2012; Zuccarello et al. 2006).

CHAPTER 6: CONCLUSIONS

6.1 Conclusions

6.1.1 Molecular phylogenetics of *Kappaphycus* and *Eucheuma* in Malaysia

The application of genetic markers in assisting the identification of local varieties of *Kappaphycus* and *Eucheuma* in Malaysia proved successful, enabling the genetic segregation of *Kappaphycus* and *Eucheuma* species, and also phylogenetic inference on these economically important seaweeds. Samples of *Kappaphycus* and *Eucheuma* deposited in the Seaweeds and Seagrasses Herbarium of University of Malaya (KLU) are summarized in Appendix S. Despite some variations in terms of resolution, all five DNA markers (*cox*1, *cox*2, *cox*2-3 spacer, *rbc*L RuBisCO spacer) demonstrated consistent results in delineating members of the genera *Kappaphycus* and *Eucheuma*, indicating that color and morphological variations of most local varieties of these red seaweeds were not supported from a genotypic standpoint. These variations were probably influenced by strain selection by farmers, or the many environmental factors during cultivation.

Even though a majority of local varieties of *Kappaphycus* and *Eucheuma* were shown to be genetically similar, molecular phylogenetics also revealed several potentially new species or cryptic species from Malaysia which are worth looking into in the future. This includes the *Kappaphycus* sp. "*Aring-aring*", *K. striatus* B1/KS1 and B2/KS2 as well as *Eucheuma denticulatum* var. *endong*. The global genetic diversity of *Kappaphycus* and *Eucheuma* was also evident based on comparisons with available GenBank sequences from elsewhere around the world. The most commonly cultivated *K. alvarezii* strain was shown to be available worldwide as a result of commercial introduction. However, another strain of *K. alvarezii*, currently unique to Africa, was also revealed, serving as a potential substitute or replacement for the dominant but

current strain which has been domesticated for four decades. The obvious genetic differences of the Hawaiian *Kappaphycus* "*alvarezii*" from the commercial strain refute the claim that all Hawaiian *Kappaphycus alvarezii* were originally introduced from the Philippines. It is believed that these Hawaiian seaweeds may be native to Hawaii in the first place, where their exact identity could be further clarified by solid morphological and molecular studies. As for *K. striatus*, specimens from the African region are limited at this time despite its being the type locality. It will be interesting to determine the relationship of *K. striatus* from Southeast Asia to the "original" ones. The same applies to *K. cottonii*, *K. inermis* and *K. procrusteanus*, which appeared to be relatively uncommon. The identity and taxonomic status of these carrageenophytes can only be clarified with more sampling efforts.

Molecular phylogeny has demonstrated the taxonomic complexity of the genus *Eucheuma*, which was shown to be paraphyletic with the positioning of *E. isiforme* samples as a sister clade to *Kappaphycus*, *Betaphycus* and other *Eucheuma*. Sampling efforts in Malaysia resulted in merely *E. denticulatum* (*E. spinosum*) and *E. denticulatum* var. *endong* Trono & Ganzon-Fortes which suggested their dominance in the Malaysian seas. The latter was shown to be conspecific to the "*Cacing*" variety of Malaysia. Results based on the near full length mitochondrial *cox*1 indicated the possibility that the *E. denticulatum* var. *endong* is currently at the stage of incipient speciation, which might explain the distinct morphological traits from *E. denticulatum* (Burman) Collins & Hervey but relatively low interspecific genetic divergence seen in most genetic markers. Again, the taxonomy pertaining to *Eucheuma* (and perhaps the closely related *Betaphycus*) can only be ascertained with wider (or deeper) samplings, which will inevitably require collaboration between countries to achieve.

Although most local *Kappaphycus* varieties in Malaysia i.e. *Buaya*, *Tambalang*, *Tangan-tangan* were shown to be conspecific; molecular differentiation has grouped

other varieties as different species i.e. wild *Flower* varieties as *K. striatus* and "*Aring-aring*" as a potentially new *Kappaphycus* species. This rejects the Null Hypothesis that the local varieties of *Kappaphycus* in Malaysia are conspecific as it is not entirely true. The Alternative Hypothesis is accepted, where local varieties of *Kappaphycus* are indeed non-conspecific to each other. Similarly for *Eucheuma*, both molecular and putative morphological results have revealed the "*Spinosum*" and "*Cacing*" varieties of *E. denticulatum* to be genetically different, again rejecting the Null Hypothesis that local varieties of *Eucheuma* are conspecific. The Alternative Hypothesis stating that local *Eucheuma* varieties are not conspecific is thus accepted.

6.1.2 Molecular marker assessment for DNA barcoding of *Kappaphycus* and *Eucheuma*

Assessments on the mitochondrial *cox*1, *cox*2, *cox*2-3 spacer and the plastid *rbc*L has shown that the *cox*2 genetic marker offered the necessary attributes as a good DNA barcode for *Kappaphycus* and *Eucheuma*. With its moderately high interspecific divergences and no intraspecific variations, the *cox*2 marker returned the highest accuracy when gauged using both the distance and tree-based DNA identification criteria. However, the popularity and relatively more established genetic database of the *cox*2-3 spacer (which is not too far off in terms of identification accuracy) rendered it a more logical candidate for the DNA barcoding of *Kappaphycus* and *Eucheuma*, particularly so when practicality and financial feasibility are taken into account.

Molecular marker assessment also displayed the genetically variable and relatively conserved traits of the mitochondrial-encoded *cox*1 and plastid-encoded *rbc*L respectively. When used individually, these markers pose some slight setbacks in terms of phylogenetic resolution and species delineation. However, when combined, these markers could potentially be good DNA barcodes for wider taxa coverage i.e the Rhodophytes.

The Large Dataset Assessment conducted in this dissertation has also shown that the incorporation of larger amounts of DNA sequences from various related species will undoubtedly decrease the extent of the Barcoding "Gap", or increase the overlaps between inter- and intraspecific genetic divergences. This will lead to an inevitable decrease in terms of identification accuracy, even so when the taxon involved is diverse in terms of species and genetic diversity. However, this decrement in identification accuracy by DNA barcoding is expected to reduce when better systematics and more complete DNA barcoding libraries are established.

The gauging of molecular markers *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L has shown that they are potential DNA barcodes for *Kappaphycus* and *Eucheuma*. These molecular markers were all capable of phylogenetic resolution and adequate species identification. Simulation of the efficiency of DNA barcodes on incorporation of large datasets of *Kappaphycus* and *Eucheuma* has also revealed the feasibility of DNA barcoding for these red algae. Based on the results of this study, the more universally used mitochondrial-encoded *cox*2-3 spacer DNA marker is recommended as the DNA barcode for *Kappaphycus* and *Eucheuma*. This rejects the Null Hypothesis that DNA barcoding is not suitable for *Kappaphycus* and *Eucheuma*. The Alternative Hypothesis is accepted, where DNA barcoding can be applied for *Kappaphycus* and *Eucheuma*.

6.2 Significant observations and appraisals of this study

This study is generally successful, providing valuable insights in aspects ranging from sample distribution and diversity, sample collection and processing, morphology, DNA extraction and amplification, phylogenetic understanding to the effectiveness of DNA barcoding on *Kappaphycus* and *Eucheuma*. The following summarizes significant observations throughout the course of the study:

1. Sample preservation requires extra care due to the gradual "sweating" effect of *Kappaphycus* and *E. denticulatum*. Constant changing of C-Fold towels is required to ensure dryness and avoid fungal growth. Only a small amount of dried, starting material (preferably ~0.5 cm length of a tip) is required for DNA extraction. Excessive starting materials will result in gelling during incubation at 65˚C, thus preventing proper homogenization.

2. The mitochondrial *cox*2 genetic marker was successfully amplified using the primers designed in this study i.e. *Kcox*2_F71 and *Kcox*2_R671. The resulting amplicons of 575bp showed good phylogenetic resolution for both *Kappaphycus* and *Eucheuma* and is thus suitable for the systematics of these red seaweeds. This marker showed intermediate levels of interspecific genetic divergence and no intraspecific variations, and displayed decent identification accuracies when gauged under the distance- and tree-based DNA identification criteria, thus a promising DNA barcode for *Kappaphycus* and *Eucheuma*.

3. The mitochondrial-encoded *cox*1 gene, at its near full length of ~1,400 bp was shown to display the highest amount of genetic variability, capable of providing the most in terms of phylogenetic resolution. The plastid *rbc*L and RuBisCO spacer, on the contrary, are the most conserved in terms of genetic variations. However, the protein-coding properties of the *rbc*L gene render it useful for genetic comparisons across wider

taxa, which is not achievable by the non-coding RuBisCO spacer. The also mitochondrial *cox*2 and *cox*2-3 spacer displayed intermediate properties in terms of resolving power for phylogeny.

4. The three main commercially cultivated carrageenophytes in Malaysia consist of *Kappaphycus alvarezii*, *K. striatus* and *Eucheuma denticulatum*. However, the biodiversity of these red algae are higher when considering wild populations. This includes *K. striatus* B2/KS2, *Kappaphycus* sp. "*Aring-aring*" and *E. denticulatum* var. *endong*, which appear to be the more dominant species or strains in the seas of Sabah. The newly introduced *K. alvarezii* in Pulau Pangkor was shown to be genetically similar to those in Sabah, indicating that they were originally cultivars from Sabah.

5. Wild and fertile specimens of *K. alvarezii* appeared to be uncommon in the seas of Sabah even though the type specimen was obtained from around the Karindingan Islands, Sabah. Wild and cystocarpic specimens of *K. striatus* B1/KS1 and *K. cottonii* appeared to be rare as well. *K. inermis* and *K. procrusteanus* were not observed in this study and are to date, unrecorded in Malaysia. *E. denticulatum* seaweeds appeared to be the dominant Eucheumatoids in Malaysian waters. On the other hand, wild and cystocarp-bearing seaweeds of *Kappaphycus* sp. "*Aring-aring*" and *K. striatus* B2/KS2 appeared to be very common in the Southeastern seas of Sabah.

6. Morphological characteristics are not really reliable in distinguishing within species of *Kappaphycus* and *Eucheuma*. However, differentiating between species can be achieved based on morphology, provided the specimens of interest are undamaged, large and mature enough with distinguishing characters. The three main morphological criteria usable for species identification include the branch diameter, branching patterns and the texture of the thalli. These criteria hold true for wild specimens too, although detailed examinations are still required for uncommon species.

7. Although local varieties of *Kappaphycus* and *Eucheuma* can be categorized together based on descriptions by local farmers, most of these local names were not valid and inaccurate, as shown by molecular results. A majority of the local varieties of *K. alvarezii* i.e. *Buaya*, *Tambalang* and *Tangan-tangan* were shown to be of the same species despite variations in terms of color and morphology, which may be attributed to the varying environmental conditions. The *Flower* varieties were shown to be conspecific to *K. striatus*, whereas the "*Aring-aring*" variety (originally thought to be *K. alvarezii*) was shown to be a distinct species. Local "*Spinosum*" and "*Cacing*" varieties on the other hand corresponded to *E. denticulatum* (Burman) Collins & Hervey (*E. spinosum*) and *E. denticulatum* (Burman) Collins & Hervey var. *endong* Trono & Ganzon-Fortes respectively.

8. Comparisons with non-local specimens of *Kappaphycus* and *Eucheuma* revealed the intrinsic diversity of these seaweeds. A sister taxa of *K. alvarezii* exclusive to Africa was revealed based on the more complete *cox*2-3 spacer dataset. The *Kappaphycus* "*alvarezii*" samples reported from Hawaii, *Kappaphycus striatus* B2/KS2 and *Kappaphycus* sp. "*Aring-aring*" were also revealed to be genetically dissimilar from one another. These species or strains may serve as potential substitutes for the commercial strain of *K. alvarezii* and also as research materials for the betterment of growth rates and carrageenan yield in the future.

9. The genus *Eucheuma* was shown to be paraphyletic, thus requiring more detailed studies. As of now, phylogenetics has also shown the relatively limited diversity of *Eucheuma* in the oceans of Malaysia. It is expected that more *Eucheuma* species are yet to be encountered or discovered around Southeast Asia considering the many reports by earlier studies.

10. Owing to the high mutation rate of the *cox*1 gene and also the relatively conserved *rbc*L gene, the genetic diversity of Rhodophytes may be over- or underestimated when these markers are used individually. It is however expected that the combined usage of both genetic markers i.e. concatenate DNA sequences would provide a better representative of the genetic compositions of these red algae, useful for the case of phylogenetics or DNA barcoding.

11. An increase in dataset will undoubtedly reduce the Barcoding "Gap" or increase the overlaps between inter- and intraspecific genetic divergences, thus affecting the overall accuracy of DNA Barcoding, especially when unidentified or wrongly identified samples are included. However, the extent of change in terms of inter- and intraspecific genetic differences varies from one DNA barcode to the other. Ultimately, the accuracy of DNA barcoding still depends largely on the size of DNA barcode libraries with proper taxonomic classification.

Despite the many important findings, this study is not without flaws. One of the biggest weaknesses of this study is the lack of specimen coverage. Although virtually all local varieties of *Kappaphycus* and *Eucheuma* were covered, wild specimens were very limited. This can be ascribed to issues associated with logistics and seasonality of these seaweeds. The long distance between University of Malaya and Semporna, Sabah means frequent sampling trips are not possible. Additionally, knowledge gaps on the distribution and seasonality of wild (preferably fertile) seaweeds of *Kappaphycus* and *Eucheuma* also render the planning and execution of field trips less effective. Efforts in looking for wild, type *K. alvarezii* (Doty) around the Karindingan Island have been futile. This hampers advancement in terms of taxonomy for *Kappaphycus* as there is to date, no DNA data of the type *K. alvarezii* (type species of *Kappaphycus*) despite its worldwide cultivation. Attempts in sequencing type *Kappaphycus* herbaria were unsucessful as well. Without reliable reference sequences of types for comparison,

description of new species or taxonomic reformations will be much more difficult. Apart from *Kappaphycus*, the species diversity of *Eucheuma* was surprisingly low as well. This might be due to difference in terms of natural habitats e.g. *Eucheuma* may be growing in deeper waters.

As a result of time constraint, it was not possible to include the description of *Kappaphycus* sp. "*Aring-aring*" as a new species in this study. Although solid molecular results have supported the genetic distinctiveness of this particular variety of *Kapppaphycus*, detailed data on morphology and distribution were not yet available. This was also true for data on *K. striatus* B2/KS2, where wild, non-fertile specimens or cultivars were not encountered and thus, incomplete. Studies on the carrageenan quality and spore cultures of each local variety of *Kappaphycus* and *Eucheuma,* although valuable, were also not conducted during the span of this study. These studies will require larger investments in terms of facilities, time and labour. However, preliminary trials on spore cultures have returned favorable results which are worth looking into in the future.

6.3 Future studies on *Kappaphycus* and *Eucheuma*

The application of molecular taxonomy to the commercially important *Kappaphycus* and *Eucheuma* has provided valuable insights for the industry. Phylogenetic delineation of *Kappaphycus* and *Eucheuma* species will allow farmers to plant different cultivars separately i.e. the isolation of "*Aring-aring*" and "*Cacing*" varieties from *K. alvarezii* to avoid decrease in carrageenan yields. The utilization of genetic markers also paved a way for tagging strains or species of commercial value for further research or domestication. The *K. alvarezii* unique to Africa, *Kappaphycus* sp. exclusive to Hawaii, *K. striatus* B2/KS2 and *Kappaphycus* sp. "*Aring-aring*" are among the interesting species or strains which can be subjected to further research such as comparative studies on growth rate, carrageenan yields as well as the characterization of carrageenan types and bioactive compounds. The documentation of *Kappaphycus* and *Eucheuma* haplotypes will also enable biomonitoring, especially when new species or strains are introduced into Malaysia which might become bioinvasive.

The genetic documentation of *Kappaphycus* and *Eucheuma* offers a general picture on the biodiversity and genetic diversity of seaweeds in Malaysia, with many information gaps yet to be filled, such as the availability of *K. cottonii*, *K. inermis*, *K. procrusteanus*, *E. arnoldii* etc. in the waters of East Malaysia. The rarity of wild and fertile *K. alvarezii* and *K. striatus* B1/KS1 is also worth looking into, particularly so for the former where Sabah is its type locality. The sampling and documentation of solid morphology and genetic data of these uncommon species of carrageenophyte will greatly assist in the systematics and also DNA barcode library establishment of *Kappaphycus* and *Eucheuma*. Also, the lack of genetic data for the taxonomically accepted species of *Kappaphycus* and *Eucheuma* will require better DNA sequencing efforts of type specimens or even designation of neotypes if needed for more accurate phylogenetic inferences and DNA barcoding. All these information gaps will definitely

require more extensive efforts in sampling which will hopefully address these issues with time.

The presence of wild and cystocarpic-bearing seaweeds of *Kappaphycus* and *Eucheuma* in Sabah serves as a good opportunity to look into *in vitro* spore cultures of these commercially significant carrageenophytes. Sexual reproduction will generate offspring with different genetic variability and vigor, which might be good replacements for the current *K. alvarezii* cultivar that has been vegetatively propagated for almost forty years. The establishment of proper spore culture collections will also pave the way for future studies on hybrid formation as well as life cycle studies.

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LIST OF PUBLICATIONS ARISING FROM THIS RESEARCH

Publications

1. Tan J, Lim PE, Phang SM (2013) Phylogenetic relationship of *Kappaphycus* Doty and *Eucheuma* J. Agardh (Solieraceae, Rhodophyta) in Malaysia. Journal of Applied Phycology 25 (1):13-29
2. Tan J, Lim PE, Phang SM, Hong DD, Sunarpi H, Hurtado AQ (2012) Assessment of Four Molecular Markers as Potential DNA Barcodes for Red Algae *Kappaphycus* Doty and *Eucheuma* J. Agardh (Solieriaceae, Rhodophyta). PLoS ONE 7 (12):e52905. doi:10.1371/journal.pone.0052905

Papers presented

1. Tan J., Lim P. E. and Phang S. M. (2012). Phylogenetic relationship of commercially important *Kappaphycus* Doty and *Eucheuma* J. Agardh in Malaysia. 2nd Conference for Regional Cooperation in Ocean and Earth Science Research in the South China Sea, University of Malaya, Kuala Lumpur, Malaysia, Oct 21-24, 2012.
2. Tan J., Lim P. E. and Phang S. M. (2011). Molecular taxonomy and genetic diversity of commercially important species of *Kappaphycus* and *Eucheuma* in Malaysia. The 6th Asian Pacific Phycological Forum (APPF 2011), Yeosu, Korea, Oct 9-14, 2011.
3. Tan J., Lim P. E. and Phang S. M. (2011). Phylogenetic Relationships of *Kappaphycus* and *Eucheuma* in Malaysia. Majlis Perlancaran-Showcase Rumput Laut Negara dan Seminar Rumpai Laut Kebangsaan 2011, Hotel Promenade, Tawau, Sabah, Apr 28-29, 2011.

4. Tan J. (2011). Phylogenetic analysis of *Kappaphycus* spp. and *Eucheuma* spp. Institute of Biological Sciences Postgraduate Seminar, University of Malaya, Aug 17, 2011.
5. Tan J. (2012). Assessment of four molecular markers as potential DNA barcodes for red algae *Kappaphycus* Doty and *Eucheuma* J. Agardh (Solieriaceae, Rhodophyta). Institute of Biological Sciences Postgraduate Seminar, University of Malaya, Dec 21, 2012.

APPENDICES

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....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70
AACTCCCATA ATGGAAGGTA TTATAAACTT ACATCATGAT TTAATGTTTT TTATTTGTGT AATTTCTATT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         80         90        100        110        120        130        140
TTTGTTTCTT GAATGTTAGG ACGTACTTTA TGACATTTTG AAAAAAATCA GAATCCTATA CCTTCTTCGT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        150        160        170        180        190        200        210
TAACTCACGG AACTTTAATA GAAATGATTT GAACTATAAC ACCAGCTTTA ATTCTTTTAA TTATAGCAAT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        220        230        240        250        260        270        280
ACCATCTTTT TCTTTATTAT ATGCAATGGA TGAAATTATA TCTCCAGCTA TAACAATTAA AACGTTAGGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        290        300        310        320        330        340        350
CATCAATGAT ATTGAAGTTA TGAATATTCT GATTATATTA ATGAAAATGA TGAAACTATA AATTTTGATA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        360        370        380        390        400        410        420
GTTATATGAT ACCTGAAGAA GATTTAGAGA AAGGTCAATT AAGGCTATTA GAGGTTGATA ATCGTATGGT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        430        440        450        460        470        480        490
GATACCTATA AATACTCATA TACGTATTAT AGTAACTGGT GCTGATGTAT TACATAGTTG AGCTGTACCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        500        510        520        530        540        550        560
TCTTTAGGAA TTAAATGTGA TGCTATTCCT GGAAGATTAA ATCAAGCTTC TCTCTTTATT AAAAGAGAAG

....|....| ....|
        570
GTATTTATTA TGGCC
```

Appendix A: Mitochondrial-encoded *cox*2 nucleotide sequence of *Solieria* sp. 120. Each dot "." represents a nucleotide position on the gene ruler, whereas each line "|" indicate a five nucleotide interval.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70
AACTCCAATT ATGGAAGGTA TTATAAATTT ACATCATGAT TTAATGTATT TTATTTGTGT AATTTTTATA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         80         90        100        110        120        130        140
TTTGTCTCTT GAATACTAGT TCGTACATTA TGACATTTTG AAAACACACA AAATACTGTA CCTTCATCAT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        150        160        170        180        190        200        210
TAGTCCATGG AACGTTAATT GAAGTTATTT GAACAGTAAC ACCTGCTTGT ATTTTGTTAA TTATCGCAAT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        220        230        240        250        260        270        280
ACCTTCATTT TCTCTTTTAT ATGCTATGGA TGAAATAATA TCTCCAGCTA TAACTATAAA AACGCTAGGT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        290        300        310        320        330        340        350
CATCAATGAT ATTGAAGTTA TGAGTATTCA GATTATTTAA ATACTGAAGG AGAATCTATT ACTTACGATA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        360        370        380        390        400        410        420
GTTATATGAT TCCTGAAGAA GATTTGAGCT TAGGACAATT AAGATTATTA GAAGTAGATA GTCGAATGGT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        430        440        450        460        470        480        490
TGTACCCGTA AATACTCATA TTCGTGTTAT TGTATCAGCA GCCGATGTGC TTCATAGTTG AGCAATACCC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        500        510        520        530        540        550        560
TCGCTAGGTA TAAAGTGTGA TGCTGTACCT GGACGTTTAA ATCAAACATC TTTATTTATT AAAAGAGAAG

....|....| ....|
        570
GTATCTATTA TGGCC
```

Appendix B: Mitochondrial-encoded *cox*2 nucleotide sequence of *Gracilaria changii* 98U. Each dot "." represents a nucleotide position on the gene ruler, whereas each line "|" indicate a five nucleotide interval.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70         80         90
TCCCATATGC TAAAATGGGA TATTGGGATC CTGAATATGT AGTTAAAGAC ACTGATGTAC TAGCTTTATT TCGCGTAAGT CCACAACCTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        100        110        120        130        140        150        160        170        180
GTGTTGACCC AATTGAAGCT TCTGCAGCTG TTGCAGGTGA ATCATCTACT GCTACTTGGA CAGTTGTTTG GACAGATCTT TTAACTGCTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        190        200        210        220        230        240        250        260        270
GTGATTTATA TAGAGCAAAG GCGTATAAAG TAGATGCTGT ACCTAATACG TCTGATCAAT ATTTTGCTTT TATTGCTTAT GATATTGACC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        280        290        300        310        320        330        340        350        360
TTTTTGAAGA AGGTTCGATT GCAAACTTGA CAGCATCAAT TATTGGTAAC GTTTTTGGGT TTAAAGCTGT AAAAGCATTA AGATTAGAAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        370        380        390        400        410        420        430        440        450
ATATGCGTAT ACCAGTAGCT TATCTAAAAA CTTTCCAAGG TCCTGCAACA GGTTTAGTTT CTGAACGTGA GCGTATGGAT AAATTTGGAC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        460        470        480        490        500        510        520        530        540
GTCCATTTTT AGGTGCAACT GTTAAGCCTA AATTAGGTTT ATCTGGTAAA AACTATGGTC GTGTAGTATA TGAAGGTCTT AAAGGTGGTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        550        560        570        580        590        600        610        620        630
TAGACTTCTT GAAAGATGAT GAAAATATCA ACTCTCAACC TTTTATGCGT TGGAAAGAAA GATATTTATA TGCTATGGAA GGCGTTAACA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        640        650        660        670        680        690        700        710        720
GATCTATTGC TGCTACAGGT GAAGTTAAAG GACATTATTT AAATGTAACT GCTGCAACAA TGGAAGATAT GTATGAGAGA GCTGAGTTCG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        730        740        750        760        770        780        790        800        810
CTAAACAGCT TGGTACTGTA ATTATCATGA TTGACCTTGT AATTGGTTAT ACTGCAATCC AAACTATGGG TATTTGGGCA CGTAAAAATG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        820        830        840        850        860        870        880        890        900
ATATGATTCT TCATTTACAC CGTGCAGGTA ACTCTACATA TTCTCGTCAA AAAATACATG GTATGAACTT CCGTGTAATT TGTAAGTGGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        910        920        930        940        950        960        970        980        990
TGCGTATGGC TGGTGTAGAC CATATTCATG CAGGTACTGT AGTAGGTAAA TTAGAAGGTG ATCCTTTAAT GATCAGAGGA TTCTATAATA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1000       1010       1020       1030       1040       1050       1060       1070       1080
CTTTATTATT ACCATATTTG AAAGTTAATC TACCTCAAGG TATCTTCTTT GAGCAAGACT GGGCATCTCT ACGTAAAGTT ACTCCAGTTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1090       1100       1110       1120       1130       1140       1150       1160       1170
CATCAGGGGG TATTCATTGT GGTCAAATGC ACCAGTTATT AGATTATCTT GGTAATGACG TTGTACTTCA ATTTGGTGGA GGTACTATTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1180       1190       1200       1210       1220       1230       1240       1250       1260
GGCATCCTGA TGGTATTCAA GCAGGTGCAA CAGCTAACCG TGTAGCTTTA GAATCAATGG TTCTAGCGCG TAATGAAGGT CGCGACTATG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1270       1280       1290       1300       1310       1320       1330       1340       1350
TTGCAGAAGG ACCGCAAATT TTACAAGACG CAGCTAAAAC TTGCGGTCCT CTACAAACAG CTCTAGATTT ATGGAAAGAT ATTACTTTTA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1360       1370       1380       1390       1400       1410       1420       1430       1440
ACTATACTTC TACAGATACT GCTGACTTCG TAGAAACTCC AACGGCTAAC GTTTAAATAA TTTTGGTTTT TATTTAGATC ATATACTAGA

....|....| ....|....| ....
       1450       1460
ATTAATACAA GAAGATAAAA GATC
```

Appendix C: Plastid-encoded *rbc*L nucleotide sequence of *Solieria* sp. 120. Each dot "." represents a nucleotide position on the gene ruler, whereas each line "|" indicate a five nucleotide interval.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70         80         90
TTCCATATGC AAAAATGGGA TACTGGGACC CTAATTATGT AGTTAAAGAT ACAGATGTAT TAGCTTTATT TCGTGTTAGT CCTCAACCAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        100        110        120        130        140        150        160        170        180
GAGTTGACCC AATAGAAGCT TCTGCTGCAG TTGCAGGTGA ATCATCTACT GCTACTTGGA CTGTTGTATG GACAGATTTA TTAACAGCTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        190        200        210        220        230        240        250        260        270
GCGACCTATA TAGAGCTAAA GCTTATAAAG TAGATGCTGT TCCAAATACT ACAGACCAGT ATTTTGCTTT TATTGCATAT GATATAGACT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        280        290        300        310        320        330        340        350        360
TGTTTGAGGA AGGCTCAATT GCTAACTTAA CAGCTTCAAT TATTGGTAAT GTGTTTGGTT TTAAAGCAGT AAAAGCTTTA CGATTAGAAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        370        380        390        400        410        420        430        440        450
ATATGCGTAT ACCAGTTGCT TACTTAAAAA CTTTTCAAGG TCCTGCTACT GGATTAGTTG TAGAACGTGA GCGTATGGAT AAGTTTGGCC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        460        470        480        490        500        510        520        530        540
GTCCGTTTTT AGGTGCAACA GTAAAACCTA AATTAGGTCT ATCTGGTAAG AACTATGGTA GAGTTGTATA TGAAGGTCTT AAAGGTGGTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        550        560        570        580        590        600        610        620        630
TAGACTTTTT GAAAGATGAT GAAAATATTA ACTCTCAGCC TTTCATGAGA TGGAAAGAAA GATTCTTATA TTCAATGGAA GGTGTTAATA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        640        650        660        670        680        690        700        710        720
GAGCAATTGC AGCAAGTGGT GAAGTCAAAG GACATTATAT GAATGTCACA GCTGCTACCA TGGAAGATAT GTATGAAAGA GCTGAATTTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        730        740        750        760        770        780        790        800        810
CTAAGCAGCT AGGCACAGTT ATCATTATGA TTGATCTGGT AATTGGTTAT ACAGCAATTC AAACTATGGC TATATGGGCA CGTAAAAATG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        820        830        840        850        860        870        880        890        900
ATATGATTTT GCATTTACAC CGTGCTGGTA ATTCAACTTA TTCTCGTCAA AAAATTCATG GAATGAATTT TCGTGTTATT TGTAAATGGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        910        920        930        940        950        960        970        980        990
TGCGTATGGC TGGTGTAGAT CATATTCATG CAGGAACTGT AGTTGGTAAA TTAGAAGGTG ATCCACTAAT GATAAAAGGA TTTTATAATA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1000       1010       1020       1030       1040       1050       1060       1070       1080
CTTTATTATT AACGCATCTA GAAATTAATT TACCTCAAGG TATATTTTTC GAACAAGATT GGGCTTCTTT ACGTAAAGTT ACGCCTGTTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1090       1100       1110       1120       1130       1140       1150       1160       1170
CTTCAGGTGG TATCCATTGT GGCCAAATGC ATCAATTACT AGATTATCTA GGTAATGATG TTGTACTTCA ATTTGGAGGC GGTACAATAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1180       1190       1200       1210       1220       1230       1240       1250       1260
GTCATCCAGA TGGTATACAG GCTGGCGCAA CAGCTAACCG TGTAGCATTA GAAGCTATGG TATTAGCTCG TAATGAAGGC CGTGATTATG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1270       1280       1290       1300       1310       1320       1330       1340       1350
TTCCAGAAGG ACCACAAATT TTACGTGATG CTGCTAAAAC ATGTGGTCCT TTGCAAACTG CTTTAGATCT ATGGAAAGAT ATTAGTTTTA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1360       1370       1380       1390       1400       1410       1420       1430       1440
ATTATACTTC TACAGATGCA GCTGATTTTG TTGAAACTCC AACAGCTAAC GTATAAAATA TATGTATTCT TTATCTTATG ATCACATTTA

....|....| ....|....| ....
       1450       1460
TTTTCATTAA ATATAATGAA AATA
```

Appendix D: Plastid-encoded *rbc*L nucleotide sequence of *Gracilaria changii* 98U. Each dot "." represents a nucleotide position on the gene ruler, whereas each line "|" indicate a five nucleotide interval.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70
TAATTATACT TCTACAGACA CTGCTGATTT TGTAGAAACT CCAACAGCTA ATGTTTAAAT AATCCTGGTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         80         90        100        110        120        130        140
CCCAATTTAA ATCAGATCAA GAATTAATAC GAAAAGATTA AAAATCTTAT ATAATTGTTT AATTATCAAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        150        160        170        180        190        200        210
GGAGTATATA TAGTGAGATT AACACAAGGA ACTTTTTCAT TCCTACCAGA TTTAACTGAC GACCAAATTA

....|....| ....|....| ....|....| ....|....| ....|....| ...
        220        230        240        250        260
CTAAACAGAT TAATTACGCC GTATCTCAAA ACTGGGCTAT CAATATAGAA TTT
```

Appendix E: Plastid-encoded RuBisCO spacer nucleotide sequence of *Eucheuma denticulatum* 41 "*Cacing*". Each dot "." represents a nucleotide position on the gene ruler, whereas each line "|" indicate a five nucleotide interval.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70
TAATTATACT TCTACAGACA CTGCTGATTT TGTAGAAACT CCAACAGCTA ATGTTTAAAT AATCCTGGTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         80         90        100        110        120        130        140
CCCAATTTAA ATCAGATCAA GAATTAATAC GAAAAGATTA AAAATCTTAT ATAATTGTTT AATTATCAAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        150        160        170        180        190        200        210
GGAGTATATA TAGTGAGATT AACACAAGGA ACTTTTTCAT TCCTACCAGA TTTAACTGAC GACCAAATTA

....|....| ....|....| ....|....| ....|....| ....|....| ...
        220        230        240        250        260
CTAAACAGAT TAATTACGCC GTATCTCAAA ACTGGGCTAT CAATATAGAA TTT
```

Appendix F: Plastid-encoded RuBisCO spacer nucleotide sequence of *Eucheuma denticulatum* 42 "*Cacing*". Each dot "." represents a nucleotide position on the gene ruler, whereas each line "|" indicate a five nucleotide interval.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70
TAATTATACT TCTACAGACA CTGCTGATTT TGTAGAAACT CCAACAGCTA ATGTTTAAAT AATCCTGGTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         80         90        100        110        120        130        140
CCCAATTTAA ATCAGATCAA GAATTAATAC GAAAAGATTA AAAATCTTAT ATAATTGTTT AATTATCAAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        150        160        170        180        190        200        210
GGAGTATATA TAGTGAGATT AACACAAGGA ACTTTTTCAT TCCTACCAGA TTTAACTGAC GACCAAATTA

....|....| ....|....| ....|....| ....|....| ....|....| ...
        220        230        240        250        260
CTAAACAGAT TAATTACGCC GTATCTCAAA ACTGGGCTAT CAATATAGAA TTT
```

Appendix G: Plastid-encoded RuBisCO spacer nucleotide sequence of Eucheuma denticulatum 97 “Cacing”. Each dot “.” represents a nucleotide position on the gene ruler, whereas each line “|” indicate a five nucleotide interval.

	56	BOH5	44	45	454	42	97	41	V6	31	1	59	460	SIT5	GUI4
56															
BOH5	0.000709														
44	0.000709	0													
45	0.000709	0	0												
454	0.00143	0.000709	0.000709	0.000709											
42	0.0085	0.0078	0.0078	0.0078	0.00709										
97	0.0085	0.0078	0.0078	0.0078	0.00709	0									
41	0.00921	0.0085	0.0085	0.0085	0.0078	0.000709	0.000709								
V6	0.148	0.147	0.147	0.147	0.147	0.146	0.146	0.145							
31	0.148	0.147	0.147	0.147	0.147	0.146	0.146	0.145	0						
1	0.148	0.147	0.147	0.147	0.147	0.146	0.146	0.145	0	0					
59	0.148	0.147	0.147	0.147	0.147	0.146	0.146	0.145	0	0	0				
460	0.148	0.147	0.147	0.147	0.147	0.146	0.146	0.145	0	0	0	0			
SIT5	0.148	0.147	0.147	0.147	0.147	0.146	0.146	0.145	0	0	0	0	0		
GUI4	0.151	0.15	0.15	0.15	0.145	0.149	0.149	0.148	0.00425	0.00425	0.00425	0.00425	0.00425	0.00425	
105	0.15	0.149	0.149	0.149	0.148	0.147	0.147	0.147	0.0113	0.0113	0.0113	0.0113	0.0113	0.0113	0.0128
83	0.15	0.149	0.149	0.149	0.148	0.147	0.147	0.147	0.0113	0.0113	0.0113	0.0113	0.0113	0.0113	0.0128
98	0.15	0.149	0.149	0.149	0.148	0.147	0.147	0.147	0.0113	0.0113	0.0113	0.0113	0.0113	0.0113	0.0128
SIT4	0.1502	0.1495	0.15	0.15	0.149	0.148	0.148	0.147	0.012	0.012	0.012	0.012	0.012	0.012	0.0134
89	0.155	0.1545	0.155	0.155	0.154	0.153	0.153	0.152	0.0432	0.0432	0.0432	0.0432	0.0432	0.0432	0.0446
121	0.155	0.1545	0.155	0.155	0.154	0.153	0.153	0.152	0.0432	0.0432	0.0432	0.0432	0.0432	0.0432	0.0446
ZAM4	0.155	0.155	0.155	0.155	0.154	0.153	0.153	0.1523	0.0432	0.0432	0.0432	0.0432	0.0432	0.0432	0.0446
58	0.155	0.155	0.155	0.155	0.154	0.153	0.153	0.152	0.0432	0.0432	0.0432	0.0432	0.0432	0.0432	0.0446
433	0.155	0.155	0.155	0.155	0.154	0.153	0.153	0.152	0.0432	0.0432	0.0432	0.0432	0.0432	0.0432	0.0446
103	0.155	0.155	0.155	0.155	0.154	0.153	0.153	0.152	0.0432	0.0432	0.0432	0.0432	0.0432	0.0432	0.0446
V7	0.155	0.155	0.155	0.155	0.154	0.153	0.153	0.152	0.0432	0.0432	0.0432	0.0432	0.0432	0.0432	0.0446
49	0.149	0.148	0.148	0.148	0.147	0.148	0.148	0.147	0.0602	0.0602	0.0602	0.0602	0.0602	0.0602	0.0602
115	0.149	0.148	0.148	0.148	0.147	0.148	0.148	0.147	0.0602	0.0602	0.0602	0.0602	0.0602	0.0602	0.0602
93	0.149	0.148	0.148	0.148	0.147	0.148	0.148	0.147	0.0609	0.0609	0.0609	0.0609	0.0609	0.0609	0.0609
120	0.15	0.15	0.15	0.15	0.149	0.149	0.149	0.148	0.128	0.128	0.128	0.128	0.128	0.128	0.13
98U	0.208	0.208	0.208	0.208	0.207	0.207	0.207	0.206	0.189	0.189	0.189	0.189	0.189	0.189	0.188

Appendix H: Genetic distances of selected *Kappaphycus* and *Eucheuma* based on the *cox*1 genetic marker for distance-based DNA barcode assessments. Values are correct to 3 significant figures. Details of samples can be referred to in Table 4.2.

	105	83	98	SIT4	89	121	ZAM4	58	433	103	V7	49	115	93	120	98U
56																
BOH5																
44																
45																
454																
42																
97																
41																
V6																
31																
1																
59																
460																
SIT5																
GUI4																
105																
83	0															
98	0	0														
SIT4	0.000709	0.000709	0.000709													
89	0.0432	0.0432	0.0432	0.0439												
121	0.0432	0.0432	0.0432	0.0439	0											
ZAM4	0.0432	0.0432	0.0432	0.0439	0	0										
58	0.0432	0.0432	0.0432	0.0439	0	0	0									
433	0.0432	0.0432	0.0432	0.0439	0	0	0	0								
103	0.0432	0.0432	0.0432	0.0439	0	0	0	0	0							
V7	0.0432	0.0432	0.0432	0.0439	0	0	0	0	0	0						
49	0.0631	0.0631	0.0631	0.0638	0.0595	0.0595	0.0595	0.0595	0.0595	0.0595	0.0595					
115	0.0631	0.0631	0.0631	0.0638	0.0595	0.0595	0.0595	0.0595	0.0595	0.0595	0.0595	0				
93	0.0638	0.0638	0.0638	0.0644	0.0602	0.0602	0.0602	0.0602	0.0602	0.0602	0.0602	0.000709	0.000709			
120	0.129	0.129	0.129	0.1297	0.131	0.131	0.131	0.131	0.131	0.131	0.131	0.13	0.13	0.13		
98U	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.192	0.192	0.193	0.173	

Appendix H, continued: Genetic distances of selected *Kappaphycus* and *Eucheuma* based on the *cox*1 genetic marker for distance-based DNA barcode assessments. Values are correct to 3 significant figures. Details of samples can be referred to in Table 4.2.

	56	454	45	44	BOH5	42	97	41	SIT5	59	GUI4	460	V6	1	31
56															
454	0														
45	0	0													
44	0	0	0												
BOH5	0	0	0	0											
42	0.00522	0.00522	0.00522	0.00522	0.00522										
97	0.00522	0.00522	0.00522	0.00522	0.00522	0									
41	0.00522	0.00522	0.00522	0.00522	0.00522	0	0								
SIT5	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158							
59	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0						
GUI4	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0	0					
460	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0	0	0				
V6	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0	0	0	0			
1	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0	0	0	0	0		
31	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0	0	0	0	0	0	
105	0.153	0.153	0.153	0.153	0.153	0.155	0.155	0.155	0.0104	0.0104	0.0104	0.0104	0.0104	0.0104	0.0104
SIT4	0.153	0.153	0.153	0.153	0.153	0.155	0.155	0.155	0.0104	0.0104	0.0104	0.0104	0.0104	0.0104	0.0104
98	0.153	0.153	0.153	0.153	0.153	0.155	0.155	0.155	0.0104	0.0104	0.0104	0.0104	0.0104	0.0104	0.0104
83	0.153	0.153	0.153	0.153	0.153	0.155	0.155	0.155	0.0104	0.0104	0.0104	0.0104	0.0104	0.0104	0.0104
433	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383
V7	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383
103	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383
121	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383
ZAM4	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383
58	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383
89	0.157	0.157	0.157	0.157	0.157	0.158	0.158	0.158	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383	0.0383
115	0.15	0.15	0.15	0.15	0.15	0.151	0.151	0.151	0.0748	0.0748	0.0748	0.0748	0.0748	0.0748	0.0748
49	0.15	0.15	0.15	0.15	0.15	0.151	0.151	0.151	0.0748	0.0748	0.0748	0.0748	0.0748	0.0748	0.0748
93	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.073	0.073	0.073	0.073	0.073	0.073	0.073
120	0.148	0.148	0.148	0.148	0.148	0.153	0.153	0.153	0.143	0.143	0.143	0.143	0.143	0.143	0.143
98U	0.221	0.221	0.221	0.221	0.221	0.226	0.226	0.226	0.231	0.231	0.231	0.231	0.231	0.231	0.231

Appendix I: Genetic distances of selected *Kappaphycus* and *Eucheuma* based on the *cox*2 marker for distance-based DNA barcode assessments. Values are correct to 3 significant figures. Details of samples can be referred to in Table 4.2.

	105	SIT4	98	83	433	V7	103	121	ZAM4	58	89	115	49	93	120	98U
56																
454																
45																
44																
BOH5																
42																
97																
41																
SIT5																
59																
GUI4																
460																
V6																
1																
31																
105																
SIT4	0															
98	0	0														
83	0	0	0													
433	0.0383	0.0383	0.0383	0.0383												
V7	0.0383	0.0383	0.0383	0.0383	0											
103	0.0383	0.0383	0.0383	0.0383	0	0										
121	0.0383	0.0383	0.0383	0.0383	0	0	0									
ZAM4	0.0383	0.0383	0.0383	0.0383	0	0	0	0								
58	0.0383	0.0383	0.0383	0.0383	0	0	0	0	0							
89	0.0383	0.0383	0.0383	0.0383	0	0	0	0	0	0						
115	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678					
49	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0.0678	0				
93	0.0661	0.0661	0.0661	0.0661	0.0661	0.0661	0.0661	0.0661	0.0661	0.0661	0.0661	0.00174	0.00174			
120	0.137	0.137	0.137	0.137	0.141	0.141	0.141	0.141	0.141	0.141	0.141	0.00174	0.00174	0.13		
98U	0.228	0.228	0.228	0.228	0.228	0.228	0.228	0.228	0.228	0.228	0.228	0.223	0.223	0.221	0.188	

Appendix I, continued: Genetic distances of selected *Kappaphycus* and *Eucheuma* based on the *cox*2 marker for distance-based DNA barcode assessments. Values are correct to 3 significant figures. Details of samples can be referred to in Table 4.2.

	56	BOH5	45	44	454	41	97	42	1	31	V6	59	GUI4	SIT5	460
56															
BOH5	0														
45	0	0													
44	0	0	0												
454	0.00274	0.00274	0.00274	0.00274											
41	0.00822	0.00822	0.00822	0.00822	0.011										
97	0.00822	0.00822	0.00822	0.00822	0.011	0									
423	0.00822	0.00822	0.00822	0.00822	0.011	0	0								
1	0.184	0.184	0.184	0.184	0.187	0.187	0.187	0.187							
31	0.184	0.184	0.184	0.184	0.187	0.187	0.187	0.187	0						
V6	0.184	0.184	0.184	0.184	0.187	0.187	0.187	0.187	0	0					
59	0.184	0.184	0.184	0.184	0.187	0.187	0.187	0.187	0	0	0				
GUI4	0.184	0.184	0.184	0.184	0.187	0.187	0.187	0.187	0	0	0	0			
SIT5	0.184	0.184	0.184	0.184	0.187	0.187	0.187	0.187	0	0	0	0	0		
460	0.184	0.184	0.184	0.184	0.187	0.187	0.187	0.187	0	0	0	0	0	0	
105	0.179	0.179	0.179	0.179	0.182	0.182	0.182	0.182	0.00551	0.00551	0.00551	0.00551	0.00551	0.00551	0.00551
83	0.179	0.179	0.179	0.179	0.182	0.182	0.182	0.182	0.00551	0.00551	0.00551	0.00551	0.00551	0.00551	0.00551
SIT4	0.179	0.179	0.179	0.1789	0.182	0.182	0.182	0.182	0.00551	0.00551	0.00551	0.00551	0.00551	0.00551	0.00551
98	0.179	0.179	0.179	0.179	0.182	0.182	0.182	0.182	0.00551	0.005501	0.00551	0.00551	0.00551	0.00551	0.00551
58	0.179	0.179	0.179	0.179	0.182	0.182	0.182	0.182	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331
ZAM4	0.179	0.179	0.1794	0.179	0.182	0.182	0.182	0.182	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331
V7	0.179	0.179	0.179	0.179	0.188	0.188	0.188	0.188	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331
433	0.179	0.179	0.179	0.179	0.182	0.182	0.182	0.182	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331
103	0.179	0.179	0.179	0.179	0.182	0.182	0.182	0.182	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331
121	0.179	0.179	0.179	0.179	0.182	0.182	0.182	0.182	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331
89	0.179	0.179	0.179	0.179	0.182	0.182	0.182	0.182	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331
115	0.1689	0.168	0.168	0.168	0.165	0.177	0.177	0.177	0.0496	0.0496	0.0496	0.0496	0.0496	0.0496	0.0496
49	0.168	0.168	0.168	0.168	0.165	0.171	0.171	0.171	0.0496	0.0496	0.0496	0.0496	0.0496	0.0496	0.0496
93	0.165	0.165	0.165	0.165	0.162	0.168	0.168	0.168	0.0468	0.0468	0.0468	0.0468	0.0468	0.0468	0.0468
120	0.173	0.173	0.173	0.173	0.171	0.176	0.176	0.176	0.168	0.168	0.168	0.168	0.168	0.168	0.168
98U	0.287	0.287	0.287	0.287	0.285	0.29	0.29	0.29	0.284	0.284	0.284	0.284	0.284	0.284	0.284

Appendix J: Genetic distances of selected *Kappaphycus* and *Eucheuma* based on the *cox*2-3 spacer marker for distance-based DNA barcode assessments. Values are correct to 3 significant figures. Details of samples can be referred to in Table 4.2.

	105	83	SIT4	98	58	ZAM4	V7	433	103	121	89	115	49	93	120	98U
56																
BOH5																
45																
44																
454																
41																
97																
423																
1																
31																
V6																
59																
GUI4																
SIT5																
460																
105																
83	0															
SIT4	0	0														
98	0	0	0													
58	0.0331	0.0331	0.0331	0.0331												
ZAM4	0.0331	0.0331	0.0331	0.0331	0											
V7	0.0331	0.0331	0.0331	0.0331	0	0										
433	0.0331	0.0331	0.0331	0.0331	0	0	0									
103	0.0331	0.0331	0.0331	0.0331	0	0	0	0								
121	0.0331	0.0331	0.0331	0.0331	0	0	0	0	0							
89	0.0331	0.0331	0.0331	0.0331	0	0	0	0	0	0						
115	0.0496	0.0496	0.0496	0.0496	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331					
49	0.0496	0.0496	0.0496	0.0496	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0.0331	0				
93	0.0468	0.0468	0.0468	0.0468	0.0303	0.0303	0.0303	0.0303	0.0303	0.0303	0.0303	0.00275	0.00275			
120	0.168	0.168	0.168	0.168	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.148	0.148	0.146		
98U	0.278	0.278	0.278	0.278	0.287	0.287	0.287	0.287	0.287	0.287	0.287	0.278	0.278	0.275	0.267	

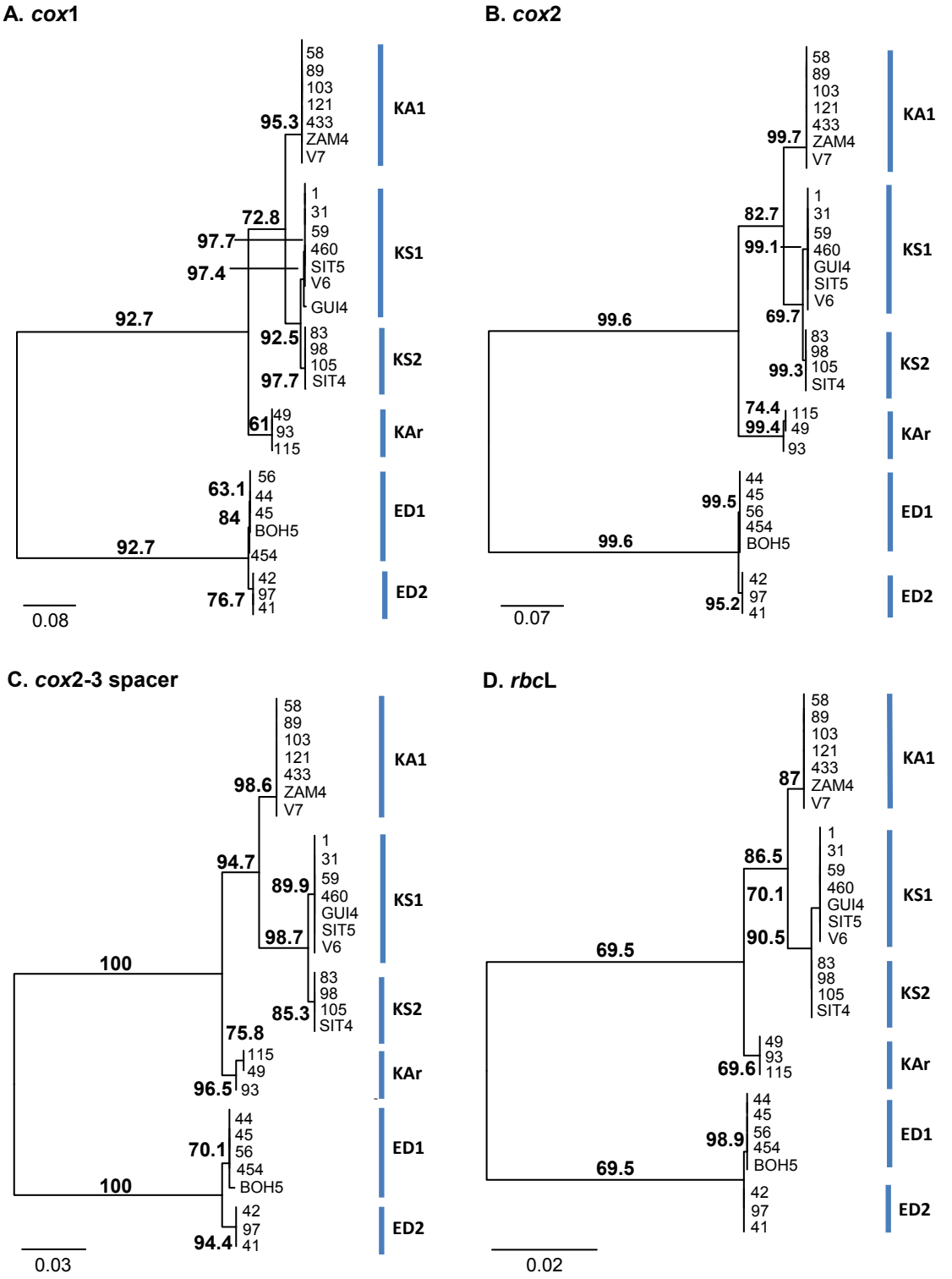
Appendix J, continued: Genetic distances of selected *Kappaphycus* and *Eucheuma* based on the *cox*2-3 spacer marker for distance-based DNA barcode assessments. Values are correct to 3 significant figures. Details of samples can be referred to in Table 4.2.

	42	97	41	44	56	45	BOH5	454	460	GUI4	31	59	1	SIT5	V6
42															
97	0														
41	0	0													
44	0.000683	0.000683	0.000683												
56	0.000683	0.000683	0.000683	0											
45	0.000683	0.000683	0.000683	0	0										
BOH5	0.000683	0.000683	0.000683	0	0	0									
454	0.000683	0.000683	0.000683	0	0	0	0								
460	0.0806	0.0806	0.0806	0.0813	0.0813	0.0813	0.0813	0.0813							
GUI4	0.0806	0.0806	0.0806	0.0813	0.0813	0.0813	0.0813	0.0813	0						
31	0.0806	0.0806	0.0806	0.0813	0.0813	0.0813	0.0813	0.0813	0	0					
59	0.0806	0.0806	0.0806	0.0813	0.0813	0.0813	0.0813	0.0813	0	0	0				
1	0.0806	0.0806	0.0806	0.0813	0.0813	0.0813	0.0813	0.0813	0	0	0	0			
SIT5	0.0806	0.0806	0.0806	0.0813	0.0813	0.0813	0.0813	0.0813	0	0	0	0	0		
V6	0.0806	0.0806	0.0806	0.0813	0.0813	0.0813	0.0813	0.0813	0	0	0	0	0	0	
105	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.00137	0.00137	0.00137	0.00137	0.00137	0.00137	0.00137
83	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.00137	0.00137	0.00137	0.00137	0.00137	0.00137	0.00137
SIR4	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.00137	0.00137	0.00137	0.00137	0.00137	0.00137	0.00137
98	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.0793	0.00137	0.00137	0.00137	0.00137	0.00137	0.00137	0.00137
58	0.0765	0.0765	0.0765	0.0772	0.0772	0.0772	0.0772	0.0772	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082
121	0.0765	0.0765	0.0765	0.0772	0.0772	0.0772	0.0772	0.0772	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082
V7	0.0765	0.0765	0.0765	0.0772	0.0772	0.0772	0.0772	0.0772	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082
103	0.0765	0.0765	0.0765	0.0772	0.0772	0.0772	0.0772	0.0772	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082
ZAM4	0.0765	0.0765	0.0765	0.0772	0.0772	0.0772	0.0772	0.0772	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082
433	0.0765	0.0765	0.0765	0.0772	0.0772	0.0772	0.0772	0.0772	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082
89	0.0765	0.0765	0.0765	0.0772	0.0772	0.0772	0.0772	0.0772	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082
115	0.0745	0.0745	0.0745	0.0752	0.0752	0.0752	0.0752	0.0752	0.015	0.015	0.015	0.015	0.015	0.015	0.015
49	0.0745	0.0745	0.0745	0.0752	0.0752	0.0752	0.0752	0.0752	0.015	0.015	0.015	0.015	0.015	0.015	0.015
93	0.0745	0.0745	0.0745	0.0752	0.0752	0.0752	0.0752	0.0752	0.015	0.015	0.015	0.015	0.015	0.015	0.015
120	0.0868	0.0868	0.0868	0.0875	0.0875	0.0875	0.0875	0.0875	0.0868	0.0868	0.0868	0.0868	0.0868	0.0868	0.0868
98U	0.16	0.16	0.16	0.159	0.159	0.159	0.159	0.159	0.162	0.162	0.162	0.162	0.162	0.162	0.162

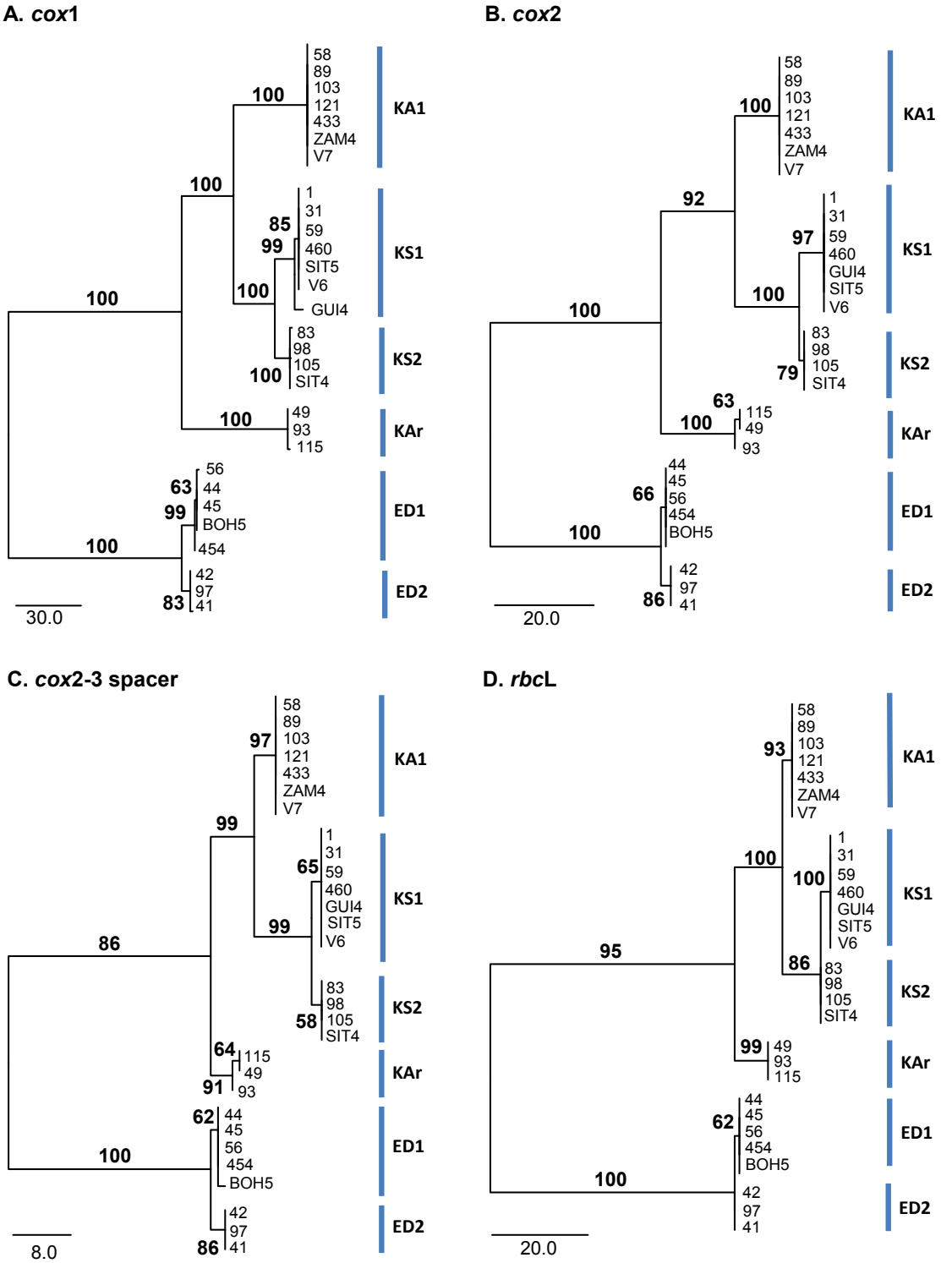
Appendix K: Genetic distances of selected *Kappaphycus* and *Eucheuma* based on the *rbc*L DNAmarker for distance-based DNA barcode assessments. Values are correct to 3 significant figures. Details of samples can be referred to in Table 4.2.

	105	83	SIT4	98	58	121	V7	103	ZAM4	433	89	115	49	93	120	98U
42																
97																
41																
44																
56																
45																
BOH5																
454																
460																
GUI4																
31																
59																
1																
SIT5																
V6																
105																
83	0															
SIR4	0	0														
98	0	0	0													
58	0.00684	0.00684	0.00684	0.00684												
121	0.00684	0.00684	0.00684	0.00684	0											
V7	0.00684	0.00684	0.00684	0.00684	0	0										
103	0.00684	0.00684	0.00684	0.00684	0	0	0									
ZAM4	0.00684	0.00684	0.00684	0.00684	0	0	0	0								
433	0.00684	0.00684	0.00684	0.00684	0	0	0	0	0							
89	0.00684	0.00684	0.00684	0.00684	0	0	0	0	0	0						
115	0.0137	0.0137	0.0137	0.0137	0.0123	0.0123	0.0123	0.0123	0.0123	0.0123	0.0123					
49	0.0137	0.0137	0.0137	0.0137	0.0123	0.0123	0.0123	0.0123	0.0123	0.0123	0.0123	0				
93	0.0137	0.0137	0.0137	0.0137	0.0123	0.0123	0.0123	0.0123	0.0123	0.0123	0.0123	0	0			
120	0.0854	0.0854	0.0854	0.0854	0.0841	0.0841	0.0841	0.0841	0.0841	0.0841	0.0841	0.0841	0.0841	0		
98U	0.162	0.162	0.162	0.162	0.158	0.158	0.158	0.158	0.158	0.158	0.158	0.158	0.158	0.149	0	

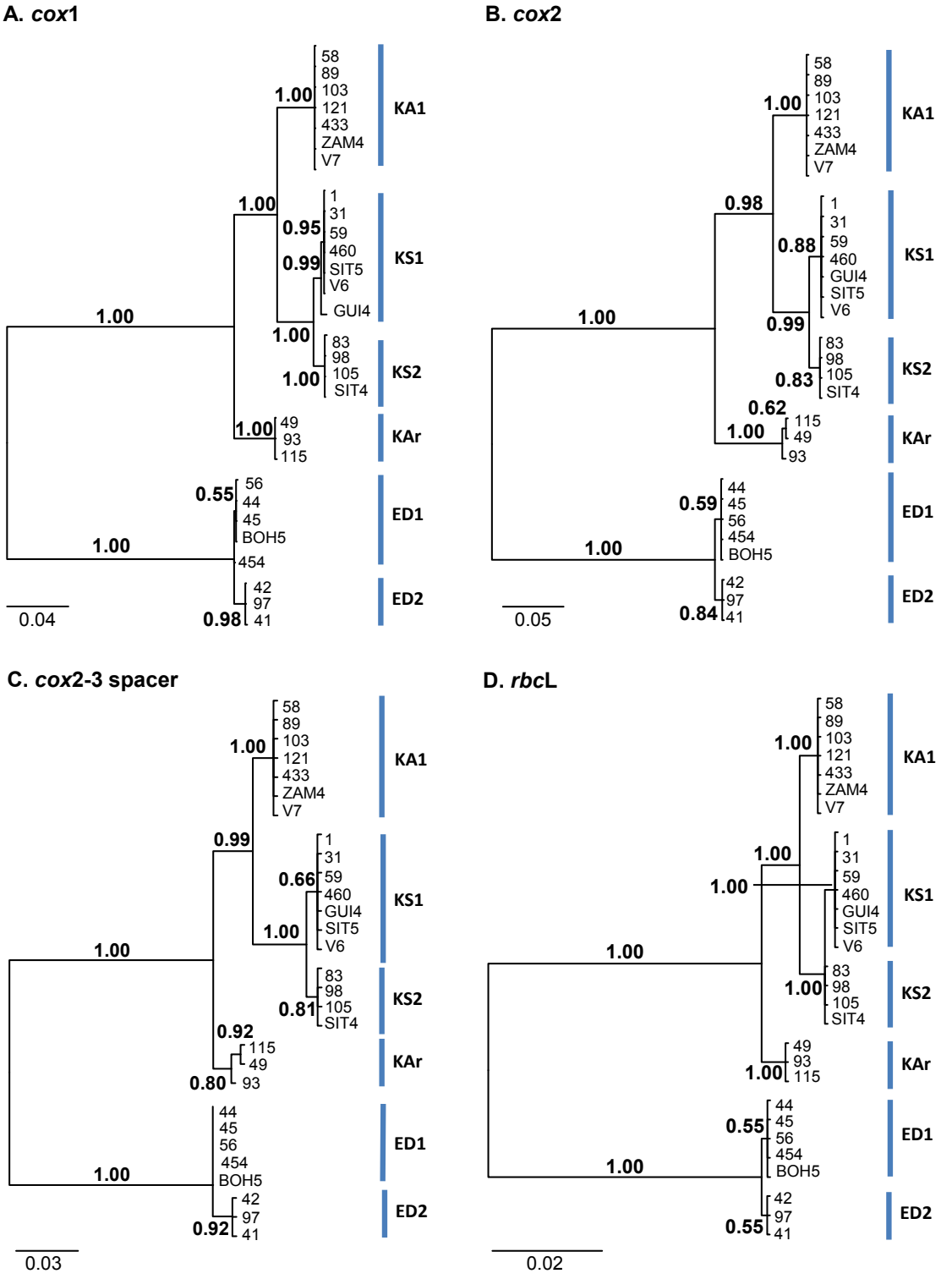
Appendix K, continued: Genetic distances of selected *Kappaphycus* and *Eucheuma* based on the *rbc*L DNAmarker for distance-based DNA barcode assessments. Values are correct to 3 significant figures. Details of samples can be referred to in Table 4.2.



Appendix L: Maximum Likelihood (ML) trees of selected *Kappaphycus* and *Eucheuma* from Southeast Asia based on (A) *cox*1; (B) *cox*2; (C) *cox*2-3 spacer; (D) *rbc*L molecular markers. Numbers at node indicate corresponding bootstrap values over 1,000 replicates. Clade annotations represent Operational Taxonomic Units (OTU), where KA= *Kappaphycus alvarezii*; KS= *Kappaphycus striatus*; KAr = *Kappaphycus* sp. "*Aring-aring*"; ED= *Eucheuma denticulatum*.

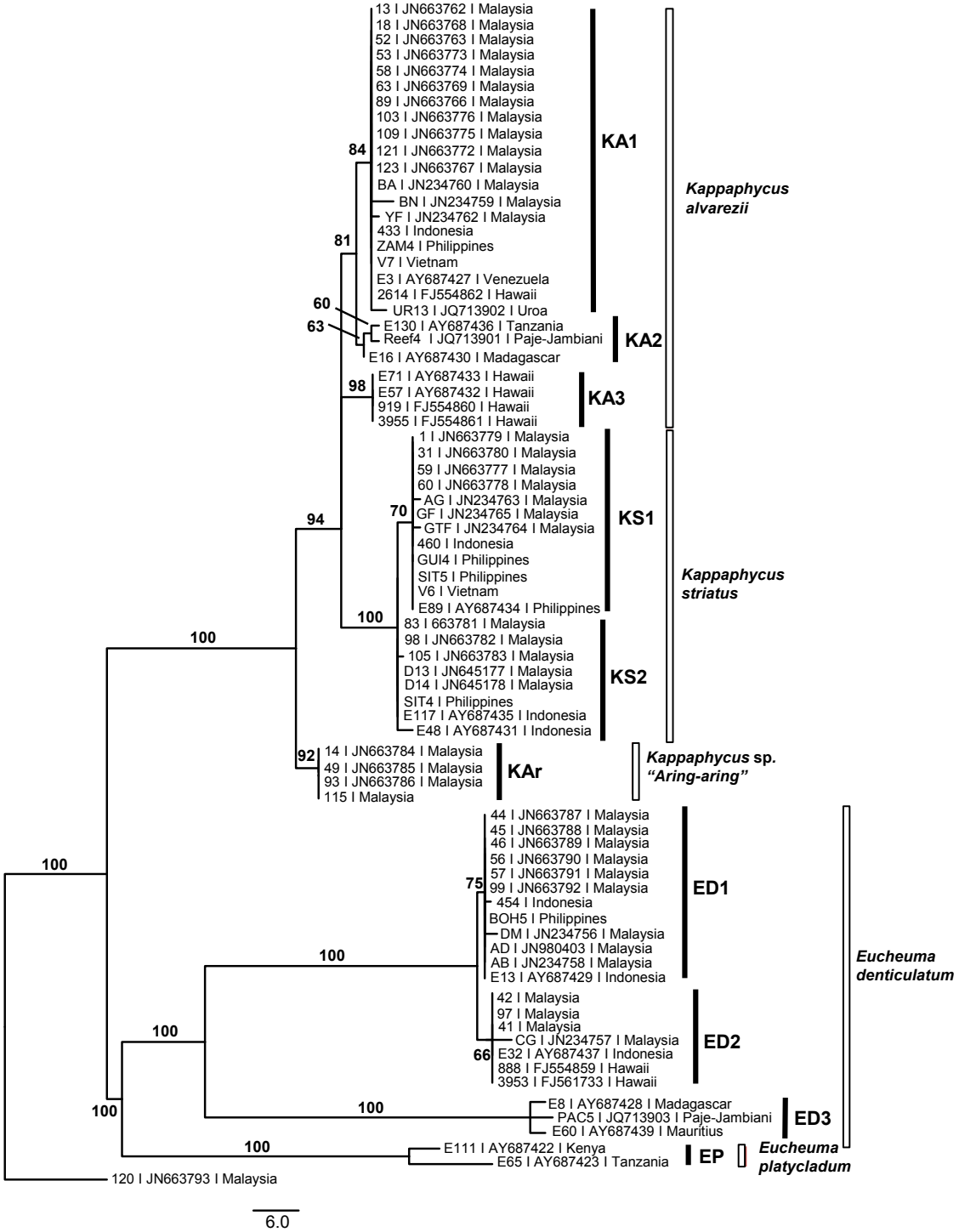


Appendix M: Maximum Parsimony (MP) trees of selected *Kappaphycus* and *Eucheuma* from Southeast Asia based on (A) *cox*1; (B) *cox*2; (C) *cox*2-3 spacer; (D) *rbc*L molecular markers. Numbers at node indicate corresponding bootstrap values over 1,000 replicates. Clade annotations represent Operational Taxonomic Units (OTU), where KA= *Kappaphycus alvarezii*; KS= *Kappaphycus striatus*; KAr = *Kappaphycus* sp. "*Aringaring*"; ED= *Eucheuma denticulatum*.

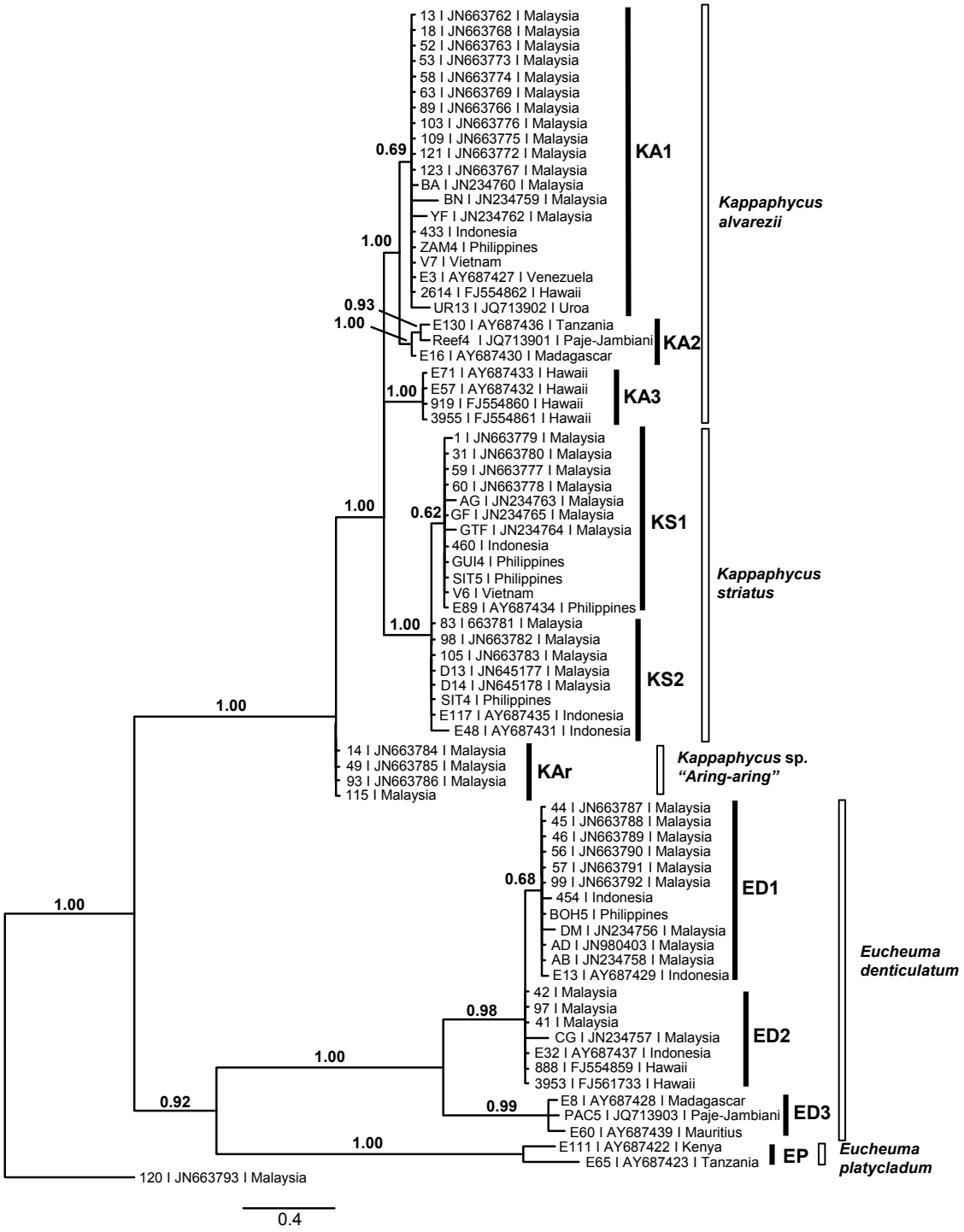


Appendix N: Bayesian Inference (BI) trees of selected *Kappaphycus* and *Eucheuma* from Southeast Asia based on (A) *cox*1; (B) *cox*2; (C) *cox*2-3 spacer; (D) *rbc*L molecular markers. Numbers at node indicate corresponding posterior probabilities over 2,000,000 generations. Clade annotations represent Operational Taxonomic Units (OTU), where KA= *Kappaphycus alvarezii*; KS= *Kappaphycus striatus*; KAr = *Kappaphycus* sp. "*Aringaring*"; ED= *Eucheuma denticulatum*.

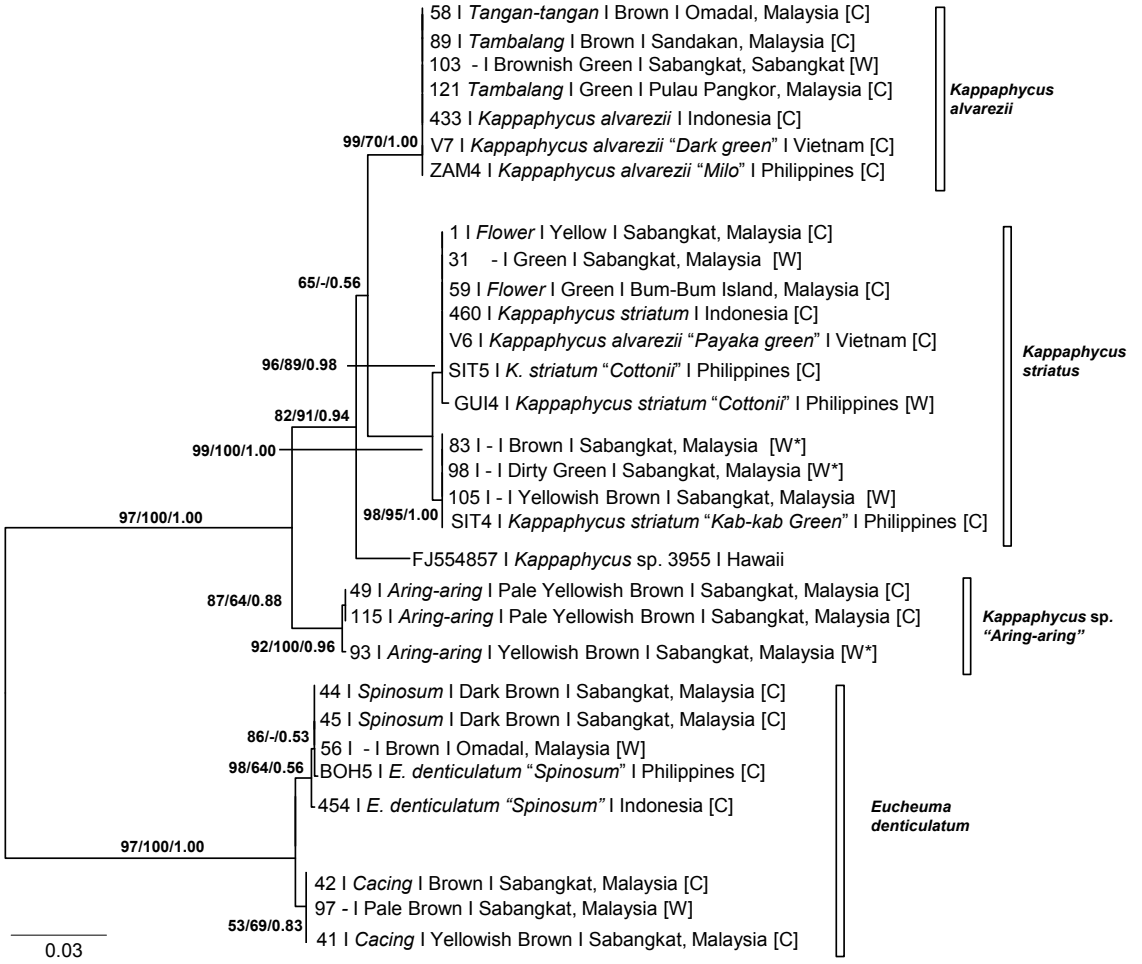
Appendix O: Neighbor-Joining (NJ) tree based on the *cox*2-3 spacer genetic marker for Large Dataset Assessment. Nodal supports indicate NJ bootstrap support over 1,000 replicates. Black bars indicate Operational Taxonomic Units (OTU), whereas white bars represent non-OTU clusters.



Appendix P: Maximum Parsimony (MP) tree based on the *cox*2-3 spacer genetic marker for Large Dataset Assessment. Nodal supports indicate NJ bootstrap support over 1,000 replicates. Black bars indicate Operational Taxonomic Units (OTU), whereas white bars represent non-OTU clusters.



Appendix Q: Bayesian Inference (BI) tree based on the *cox*2-3 spacer genetic marker for Large Dataset Assessment. Nodal supports indicate NJ bootstrap support over 1,000 replicates. Black bars indicate Operational Taxonomic Units (OTU), whereas white bars represent non-OTU clusters.



Appendix R: Maximum Likelihood 50% majority-rule consensus tree based on the combined *cox*1 and *cox*2-3 spacer genetic markers. -Ln likelihood score was -2460.058. (Substitution rate parameters: TC= 0.4046893; TA= 0.04765535; TG0.04765535; CA= 0.04765535; CG= 0.04765535; AG= 0.4046893). Nodal supports are arranged in an order of ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. Local specimens are denoted according to isolate no. | variety | color | origin | cultivated [C] or wild [W]. Non-local specimens were denoted as follows: isolate no. | sample name | origin | cultivated [C] or wild [W]. Asterisks (*) indicate cystocarpic plants.

Appendix S: Samples of *Kappaphycus* and *Eucheuma* deposited in the University of Malaya Seaweed and Seagrass Herbarium (KLU), Malaysia

No.	Name	Location	Date of Collection	Collection Code
1	1- *Kappapphycus striatus* "*Yellow Flower*" [C]	Sabangkat	21.06.2010	PSM11984-UMSS0128/0129
2	2- *Kappapphycus striatus* "*Yellow Flower*" [C]			PSM11985-UMSS0130/0131
3	3- *Kappapphycus striatus* "*Yellow Flower*" [C]			PSM11986-UMSS0132/0133
4	4- *Kappapphycus striatus* "*Yellow Flower*" [C]			PSM11987-UMSS0134/0135
5	5- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM11988-UMSS0136
6	6- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM11989-UMSS0137
7	7- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM11990-UMSS0138
8	8- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM11991-UMSS0139
9	9- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM11992-UMSS0140
10	10- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM11993-UMSS0141
11	11- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM11994-UMSS0142
12	12- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM11995-UMSS0143
13	13- *Kappaphycus alvarezii* "*Buaya*" [C]			PSM11996-UMSS0144/0145
14	14- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM11997-UMSS0146/0147
15	15- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM11998-UMSS0148/0149
16	16- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM11999-UMSS0150/151
17	17- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12000-UMSS0152/153
18	18- *Kappaphycus alvarezii* "*Giant*" [C]			PSM12001-UMSS0154
19	19- *Kappaphycus alvarezii* "*Buaya*" [C]			PSM11980-UMSS0123
20	20- *Kappaphycus alvarezii* "*Buaya*" [C]			PSM11981-UMSS0124
21	21- *Kappaphycus alvarezii* "*Buaya*" [C]			PSM11982-UMSS0125
22	22- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM12002-UMSS0155
23	23- *Kappaphycus alvarezii* "*Buaya*" [C]			PSM12003-UMSS0156
24	24- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM12004-UMSS0157
25	25- *Kappapphycus striatus* "*Green Flower*" [C]			PSM12005-UMSS0158/0159
26	26- *Kappapphycus striatus* "*Green Flower*" [C]			PSM12006-UMSS0160/161
27	27- *Kappapphycus striatus* "*Green Flower*" [C]			PSM12007-UMSS0162/0163
28	28- *Kappaphycus alvarezii* "*Tambalang Brown*" [C]			PSM12008-UMSS0164/0165
29	29- *Kappaphycus alvarezii* "*Tambalang Brown*" [C]			PSM12009-UMSS0166/167
30	30- *Kappaphycus alvarezii* "*Tambalang Brown*" [C]			PSM12010-UMSS0168/0169
31	31- *Kappapphycus striatus* [W]			PSM12011-UMSS0170

Appendix S, continued

No.	Name	Location	Date of Collection	Collection Code
32	32- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]	Sabangkat, Sabah, Malaysia	21.06.2010	PSM12012-UMSS0171
33	35- *Kappapphycus striatus* [W]			PSM12013-UMSS0172/0173/0174
34	36- *Kappapphycus striatus* [W]			PSM12014-UMSS0175/0176
35	37- *Kappaphycus alvarezii* "*Tambalang Green*" [C]			PSM12015-UMSS0177/0178
36	38- *Kappaphycus alvarezii* "*Tambalang Green*" [C]			PSM12016-UMSS0179
37	39- *Kappaphycus alvarezii* "*Tambalang Green*" [C]			PSM12017-UMSS0180/0181
38	40- *Kappaphycus alvarezii* "*Tambalang Brown*" [C]			PSM11983-UMSS0126/0127
39	41- *Eucheuma denticulatum* "*Cacing*" [C]			PSM12018-UMSS0181/0182
40	42- *Eucheuma denticulatum* "*Cacing*" [C]			PSM12019-UMSS0183/0184
41	43- *Eucheuma denticulatum* "*Cacing*" [C]			PSM12020-UMSS0185/0186
42	44- *Eucheuma denticulatum* "*Spinosum*" [C]			PSM12021-UMSS0187
43	45- *Eucheuma denticulatum* "*Spinosum*" [C]			PSM12022-UMSS0188
44	46- *Eucheuma denticulatum* "*Spinosum*" [C]			PSM12023-UMSS0189
45	47- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12024-UMSS0190
46	48- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12025-UMSS0191
47	49- *Kappaphycus* sp. "*Aring-aring*" [C]	Omadal, Sabah, Malaysia	22.06.2010	PSM12026-UMSS0192
48	50- *Kappaphycus alvarezii* "*Buaya*" [C]			PSM12027-UMSS0193
49	51- *Kappaphycus alvarezii* "*Buaya*" [C]			PSM12028-UMSS0194/0195
50	52- *Kappaphycus alvarezii* "*Buaya*" [C]			PSM12029-UMSS0196/0197
51	53- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM12030-UMSS0198
52	54- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM12031-UMSS0199
53	55- *Eucheuma denticulatum* "*Spinosum*" [W]			PSM12032-UMSS0200
54	56- *Eucheuma denticulatum* "*Spinosum*" [W]			PSM12033-UMSS0201
55	57- *Eucheuma denticulatum* "*Spinosum*" [W]			PSM12034-UMSS0202
56	58- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM12035-UMSS0203
57	59- *Kappapphycus striatus* "*Green Flower*" [C]	Bum-Bum Island, Sabah, Malaysia		PSM12039-UMSS0207/0208
58	60- *Kappapphycus striatus* "*Green Flower*" [C]			PSM12040-UMSS0209/0210
59	61- *Kappapphycus striatus* "*Green Flower*" [C]			PSM12041-UMSS0211/0212
60	62- *Kappaphycus alvarezii* "*Giant*" [C]	Sisipan, Sabah, Malaysia		PSM12042-UMSS0213
61	63- *Kappaphycus alvarezii* "*Giant*" [C]			PSM12043-UMSS0214
62	64- *Kappaphycus alvarezii* "*Giant*" [C]			PSM12044-UMSS0215

Appendix S, continued

No.	Name	Location	Date of Collection	Collection Code
63	65- *Kappaphycus* sp. "*Aring-aring*" [W*]	Omadal, Sabah, Malaysia		PSM12036-UMSS0204
64	66- *Kappaphycus* sp. "*Aring-aring*" [W]			PSM12037-UMSS0205
65	67- *Eucheuma denticulatum* "*Spinosum*" [W]			PSM12038-UMSS0206
66	68- *Kappaphycus* sp. "*Aring-aring*" [C]	Sisipan, Sabah, Malaysia		PSM12045-UMSS0216
67	69- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12046-UMSS0217
68	70- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12047-UMSS0218
69	81- *Kappaphycus* sp. "*Aring-aring*" [W*]	Sabangkat, Sabah, Malaysia	24.06.2010	PSM12051-UMSS0222
70	82- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12052-UMSS0223
71	83- *Kappaphycus striatus* [W*]			PSM12053-UMSS0224
72	84- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12054-UMSS0225
73	85- *Kappaphycus* sp. "*Aring-aring*" [W]			PSM12055-UMSS0226
74	86- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12056-UMSS0227
75	87- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12057-UMSS0228
76	88- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12058-UMSS0229
77	89- *Kappaphycus alvarezii* "*Tambalang Brown*" [C]	Sandakan, Sabah, Malaysia	08.11.2010	PSM12059-UMSS0230
78	90- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12060-UMSS0231
79	91- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12061-UMSS0232
80	92- *Kappaphycus* sp. "*Aring-aring*" [W*]	Sabangkat, Sabah, Malaysia	15.11.2010	PSM12062-UMSS0233
81	93- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12063-UMSS0234
82	94- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12064-UMSS0235
83	95- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12065-UMSS0236
84	97- *Eucheuma denticulatum* "*Cacing*" [W]			PSM12066-UMSS0237
85	98- *Kappaphycus striatus* [W*]			PSM12067-UMSS0238
86	99- *Eucheuma denticulatum* "*Spinosum*" [W]			PSM12068-UMSS0239
87	100- *Eucheuma denticulatum* "*Cacing*" [W]			PSM12069-UMSS0240
88	101- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12070-UMSS0241
89	102- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12071-UMSS0242
90	103- *Kappaphycus alvarezii* [W]			PSM12072-UMSS0243
91	104- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12073-UMSS0244
92	105- *Kappaphycus striatus* [W*]			PSM12074-UMSS0245
93	106- *Kappaphycus* sp. "*Aring-aring*" [W*]			PSM12075-UMSS0246

Appendix S, continued

No.	Name	Location	Date of Collection	Collection Code
94	107- *Kappaphycus* sp. "*Aring-aring*" [W*]	Semporna, Sabah, Malaysia	16.11.2010	PSM12076-UMSS0247
95	108- *Kappapphycus striatus* "*Yellow Flower*" [C]			PSM12077-UMSS0248
96	109- *Kappaphycus alvarezii* "*Tangan-tangan*" [C]			PSM12078-UMSS0249
97	110- *Kappaphycus alvarezii* "*Buaya*" [C]	Sabangkat, Sabah, Malaysia	29.04.2011	PSM12079-UMSS0250
98	111- *Kappaphycus alvarezii* "*Tambalang Green*" [C]			PSM12080-UMSS0251
99	112- *Kappapphycus striatus* "*Yellow Flower*" [C]	Sabangkat, Sabah, Malaysia	01.06.2011	PSM12097-UMSS0252
100	113- *Kappapphycus striatus* "*Yellow Flower*" [C]			PSM12098-UMSS0253
101	114- *Kappapphycus striatus* "*Yellow Flower*" [C]			PSM12099-UMSS0254
102	115- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12100-UMSS0255
103	116- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12101-UMSS0256
104	117- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12102-UMSS0257
105	118- *Kappaphycus* sp. "*Aring-aring*" [C]			PSM12103-UMSS0258
106	121- *Kappaphycus alvarezii* "*Tambalang Green*" [C]	Pulau Pangkor, Peninsular Malaysia, Malaysia	03.06.2011	PSM12105-UMSS0260
107	122- *Kappaphycus alvarezii* "*Tambalang Brown*" [C]			PSM12106-UMSS0261
108	123- *Kappaphycus alvarezii* "*Tambalang Brown*" [C]			PSM12107-UMSS0262
109	124- *Kappaphycus alvarezii* "*Tambalang Brown*" [C]			PSM12108-UMSS0263
110	125- *Kappaphycus alvarezii* "*Tambalang Green*" [C]	Pulau Pangkor, Peninsular Malaysia, Malaysia	12.09.2011	PSM12277-UMSS0354
111	126- *Kappaphycus alvarezii* "*Tambalang Green*" [C]			PSM12278-UMSS0355
112	127- *Kappaphycus alvarezii* "*Tambalang Green*" [C]			PSM12279-UMSS0356
113	GUI1- *Kappaphycus* sp. [W]	Guimaras Island, Panay, Philippines	02.05.2010	AQHGUI001-UMSS0357
114	GUI2 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI002-UMSS0358
115	GUI3 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI003-UMSS0359
116	GUI4 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI004-UMSS0360
117	GUI5 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI005-UMSS0361
118	GUI6 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI006-UMSS0362
119	GUI7 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI007-UMSS0363
120	GUI8 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI008-UMSS0364
121	GUI9 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI009-UMSS0365
122	GUI10 *Kappaphycus striatus* "*Cottonii*" [W]			AQHGUI010-UMSS0366
123	BOH1 *Kappaphycus alvarezii* "*Cottonii*" [C]	Bohol, Central Visayas, Philippines	16.05.2010	AQHBOH001-UMSS0367
124	BOH2 *Eucheuma denticulatum* "*Spinosum*" [W]			AQHBOH002-UMSS0368

Appendix S, continued

No.	Name	Location	Date of Collection	Collection Code
125	BOH3 *Eucheuma denticulatum* "*Spinosum*" [C]	Bohol, Central Visayas, Philippines	16.05.2010	AQHBOH003-UMSS0369
126	BOH4 *Eucheuma denticulatum* "*Spinosum*" [C]			AQHBOH004-UMSS0370
127	BOH5 *Eucheuma denticulatum* "*Spinosum*" [C]			AQHBOH005-UMSS0371
128	BOH6 *Eucheuma denticulatum* "*Spinosum*" [C]			AQHBOH006-UMSS0372
129	BOH7 *Kappaphycus striatus* "*Cottonii*" [C]			AQHBOH007-UMSS0373
130	BOH8 *Kappaphycus alvarezii* "*Cottonii*" [C]			AQHBOH008-UMSS0374
131	BOH9 *Kappaphycus alvarezii* "*Cottonii*" [W]			AQHBOH009-UMSS0375
132	ZAM1 *Kappaphycus alvarezii* "*Kab-kab*" [C]	Zamboanga City, Mindanao, Philippines	16.05.2010	AQHZAM001-UMSS0377
133	ZAM3 *Kappaphycus alvarezii* "Vanguard" [C]			AQHZAM003-UMSS0379
134	ZAM4 *Kappaphycus alvarezii* "*Milo*" [C]			AQHZAM004-UMSS0380
135	ZAM5 *Kappaphycus alvarezii* [C]			AQHZAM005-UMSS0381
136	ZAM6 *Kappaphycus alvarezii* "*Sacol*" Red [C]			AQHZAM006-UMSS0382
137	ZAM7 *Kappaphycus alvarezii* "Purple" [C]			AQHZAM007-UMSS0383
138	ZAM8 *Kappaphycus alvarezii* "Purple" [C]			AQHZAM008-UMSS0384
139	ZAM9 *Kappaphycus alvarezii* "*Milo*" [C]			AQHZAM009-UMSS0385
140	ZAM10 *Kappaphycus alvarezii* "Purple" [C]			AQHZAM010-UMSS0386
141	ZAM11 *Kappaphycus striatus* "*Brown*" [C]			AQHZAM011-UMSS0387
142	ZAM12 *Kappaphycus striatus* "*Green*" [C]			AQHZAM012-UMSS0388
143	ZAM13 *Kappaphycus striatus* "*Brownish Green*" [C]			AQHZAM013-UMSS0389
144	SIT1 *Kappaphycus striatus* "*Bitsi-bitsi Green* (Giant *kab-kab*)" [C]	Sitangkai, Tawi, Mindanao, Philippines	23.09.2010	AQHSIT001-UMSS0390
145	SIT2 *Kappaphycus striatus* "*Bitsi-bitsi Green* (Giant *kab-kab*)" [C]			AQHSIT002-UMSS0391
146	SIT3 *Kappaphycus striatus* "*Kab-kab*" Green [C]			AQHSIT003-UMSS0392
147	SIT5 *Kappaphycus striatus* "*Cottonii light green* (Sacol)" [C]			AQHSIT005-UMSS0394
148	SIT6 *Kappaphycus striatus* "*Cottonii light green* (Sacol)" [C]			AQHSIT006-UMSS0395
149	SIT7 *Kappaphycus striatus* "*Cottonii Red* (Sacol)" [C]			AQHSIT007-UMSS0396
150	SIT8 *Kappaphycus striatus* "*Cottonii Red* (Sacol)" [C]			AQHSIT008-UMSS0397
151	SIT9 *Kappaphycus striatus* "*Cottonii Brown* (Sacol)" [C]			AQHSIT009-UMSS0398
152	SIT10 *Kappaphycus striatus* "*Cottonii Brown* (Sacol)" [C]			AQHSIT010-UMSS0399
153	SIT11 *Kappaphycus alvarezii* "*Tambalang Red Brown* (Sacol)" [C]			AQHSIT011-UMSS0400
154	SIT12 *Kappaphycus alvarezii* "*Tambalang Red Brown* (Sacol)" [C]			AQHSIT012-UMSS0401

Appendix S, continued

No.	Name	Location	Date of Collection	Collection Code
155	V1 *Kappaphycus alvarezii* "Brown-Short" [C]	Cam Ranh, Khanh Hoa, Vietnam	2006	PSM12374-UMSS0519
156	V2 *Kappaphycus alvarezii* "Brown-Long" [C]			PSM12375-UMSS0520
157	V3 *Kappaphycus alvarezii* "Payaka" Brown [C]			PSM12376-UMSS0521
158	V4 *Kappaphycus alvarezii* "Payaka" Brown [C]	Nha Trang, Khanh Hoa, Vietnam		PSM12377-UMSS0522
159	V5 *Kappaphycus striatus* "Payaka" Green [C]			PSM12378-UMSS0523
160	V6 *Kappaphycus striatus* "Payaka" Green [C]	Cam Ranh, Khanh Hoa, Vietnam		PSM12379-UMSS0524
161	V7 *Kappaphycus alvarezii* "Dark Green" [C]	Son Hai, Ninh Thuan, Vietnam	-	PSM12380-UMSS0525
162	V8 *Kappaphycus alvarezii* "Brown" [C]	Cam Ranh, Khanh Hoa, Vietnam	-	PSM12381-UMSS0526
163	V9 *Kappaphycus alvarezii* "Payaka" Brown [C]	Van Ninh, Khanh Hoa, Vietnam	-	PSM12382-UMSS0527
164	V10 *Kappaphycus alvarezii* "Brown" [C]		-	PSM12383-UMSS0528
165	V11 *Kappaphycus alvarezii* [C]	Ninh Diem, Ninh Phuoc, Ninh Thuan, Vietnam	May 1999	PSM12384-UMSS0529
166	V12 *Kappaphycus alvarezii* [C]	Ninh Phuoc, Ninh Thuan, Vietnam	-	PSM12385-UMSS0530
167	V13 *Kappaphycus alvarezii* [C]	Ninh Thuan, Vietnam	Sept 1999	PSM12386-UMSS0531
168	V14 *Kappaphycus alvarezii* [C]	Phan Rang, Ninh Thuan, Vietnam	May 2007	PSM12387-UMSS0532
169	V15 "*Kappaphycus cottonii*" [W]	Truong Sa Island, Khanh Hoa, Vietnam	2000	PSM12388-UMSS0533

[1] [C] = cultivated specimens, [W] = wild specimens, [W*] = wild, cystocarpic specimens; dashes "-" indicate non-available data

[2] Samples from the Philippines and Vietnam were kindly provided by Dr. Anicia Q. Hurtado and Associate Prof. Dang Diem Hong respectively. Assignment of herbarium and/or silica gel codes is done with consent from each party.